The Mechanism of Orotidine 5′-Monophosphate Decarboxylase: Catalysis by Destabilization of the Substrate†

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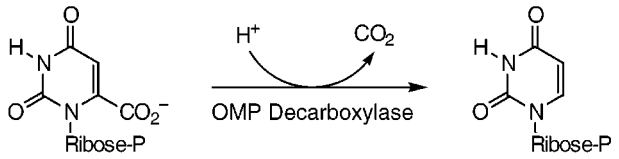
ABSTRACT: The mechanism of orotidine 5′-monophosphate decarboxylase (OMP decarboxylase, ODCase) was studied using the decarboxylation of orotic acid analogues as a model system. The rate of decarboxylation of 1,3-dimethylorotic acid and its analogues as well as the stability of their corresponding carbanion intermediates was determined. The results have shown that the stability of the carbanion intermediate is not a critical factor in the rate of decarboxylation. On the other hand, the reaction rate is largely dependent on the equilibrium constant for the formation of a zwitterion. Based on these results, we have proposed a new mechanism in which ODCase catalyzes the decarboxylation of OMP by binding the substrate in a zwitterionic form and providing a destabilizing environment for the carboxylate group of OMP.

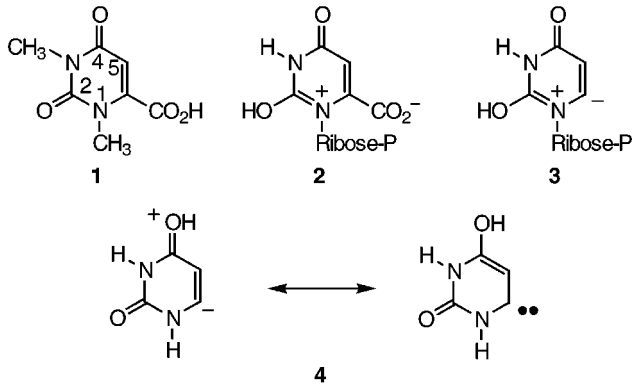
Orotidine 5′-monophosphate decarboxylase (OMP decarboxylase, ODCase)[1] catalyzes the conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP) (Scheme 1), the final step of de novo pyrimidine nucleotide biosynthesis (*1*). Unlike other decarboxylases, which stabilize the carbanion intermediates by delocalizing the negative charge into an electrophilic π-system of the substrate (*2*) or a covalently attached cofactor (*3*), ODCase contains no cofactors, and the reaction intermediate is a nonconjugated carbanion. Recently it has been suggested that Zn ions play a role in the decarboxylation of OMP (*4*). However, subsequent studies failed to detect Zn or other transition metal ions in ODCase (*5*). Despite the lack of cofactors, ODCase is a very proficient enzyme by comparison of the rate of the enzymatic decarboxylation to that of the nonenzymatic decarboxylation of orotic acid derivatives, and a rate enhancement of 1.4×10^{17}-fold has been reported (*6*).

The mechanism by which ODCase catalyzes the decarboxylation of OMP remains unknown despite significant research effort by chemists and biochemists. Three mechanisms have been proposed to explain the facile enzymatic decarboxylation (*7*−*9*). Beak and Siegel examined the nonenzymatic decarboxylation of 1,3-dimethylorotic acid (**1**) and several related compounds as models for the enzymatic reaction and proposed a zwitterion mechanism for the decarboxylation of OMP which involves zwitterion **2** and the inductively stabilized ylide **3** as intermediates in the reaction pathway (Scheme 2) (*7*). Based on quantum mechanical calculations, Lee and Houk proposed that proton transfer to the more basic oxygen-4 concerted with loss of carbon dioxide would give a more stable carbene-like intermediate (**4**) (*9*). Recent model studies in our laboratory have demonstrated the viability of this proposal which does not require a positive charge at position-1 (*10*). We have also confirmed that a carbon-6-centered nucleophile is a discrete intermediate in the model reaction, consistent with either mechanism (*11*). Other model studies led Silverman and Groziak to propose a covalent mechanism involving

Scheme 1



Scheme 2



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[1] Abbreviations: ODCase, orotidine 5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; CAD, collision-activated dissociation; ESI, electrospray ionization; PLP, pyridoxal phosphate; TPP, thiamin pyrophosphate.

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nucleophilic attack at C-5 by an active site residue in which the formation of the seemingly unstable vinylic carbanion would not be necessary (*8*). However, subsequent studies of kinetic isotope effects (*12, 13*) and substrate analogues (*14, 15*) provide a strong argument against this mechanism.

Much of the previous mechanistic work has concentrated on illustrating how the vinylic carbanion intermediate can be stabilized in the active site. We thought that the enzyme might have limited capacity to provide stabilization to the carbanion and decided to further investigate the chemical fundamentals of the model reactions. We wanted to understand whether the stability of the carbanion intermediate is a major factor in the rate of the nonenzymatic decarboxylation and, if not, what is the most important factor in determining the rate of decarboxylation of orotic acid analogues. The present paper reports mechanistic implications for the decarboxylation of OMP based on our studies on model systems.

MATERIALS AND METHODS

Kinetic Measurements. The rates of decarboxylation were determined manometrically by following the evolution of carbon dioxide using a U-tube manometer. Two reaction flasks were connected to the two sides of the U-tube and were heated to the same temperature. One flask contained the decarboxylation substrate and solvent while the other one was used as a control and was charged with solvent only. The substrate concentration for these studies was 0.17 M. The pressure of the reaction system was recorded vs reaction time. Data analysis followed the method reported by Beak and Siegel (*7*).

Chemicals. Acid **1** was prepared through the methylation of orotic acid (from Sigma) as reported by Curran and Augier (*16*). The same method was used to prepare **5** from 2-pyridone-6-carboxylic acid (from Aldrich). Acid **6** was prepared from 4-pyrone-2-carboxylic acid (from TCI America) as reported by Beak and Siegel (*7*). The identity and purity of the synthesized compounds were verified by their ^{1}H NMR spectrum, mass spectrum, and melting point.

Proton Affinity Determinations and Computational Calculations. All mass spectrometric experiments were completed in a modified Finnigan-LCQ quadrupole ion trap mass spectrometer. Ions were generated by electrospray from $10^{-4}-10^{-5}$ M solutions of the precursors in methanol using a flow rate of $3-5$ μL/min. Typically, an electrospray needle voltage of approximately 4000 V was used.

Experimental determination of the proton affinities of the carbanion intermediates and orotic acid analogues followed the methods discussed in our previous paper, and a very brief discussion is presented under Results and Discussion (*17*). Computational studies followed the procedures outlined in our earlier publication (*17*).

RESULTS AND DISCUSSION

The intriguing decarboxylation rate difference among the orotic acid analogues **1**, **5**, and **6** (Scheme 3) makes this a useful system to study (*7*). The reaction rates differ by 3 orders of magnitude, as shown in Table 1, despite their structural similarity. Since the transition state for decarboxylation likely resembles the resulting carbanion, structurally and energetically, it is possible that a more stable carbanion

Scheme 3

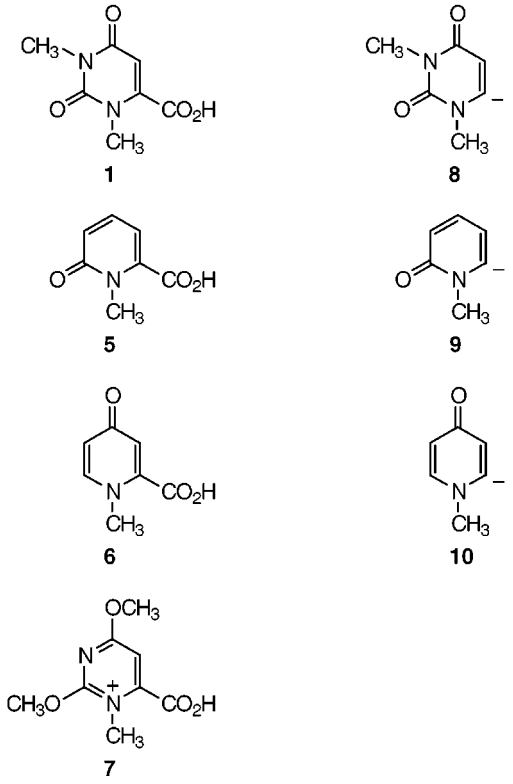


Table 1: Rate Constants for Decarboxylation of Orotic Acid Analogues at 206 °C

substrate	rate constants, s^{-1}	
	sulfolane	isoquinoline
1	7.5×10^{-4} [a]; 7.6×10^{-4} [b]	1.6×10^{-3} [a]; 1.4×10^{-3} [b]
5	1.2×10^{-3} [a]	1.3×10^{-3} [a]; 1.2×10^{-3} [b]
6	0.32[a,c]	1.6[a,c]; 3.2[b,c]
7	5.0×10^{4} [b,c]	
	1.4×10^{-6} [c,d] (25 °C)	

[a] Rate constants from the present study. [b] Rate constants from ref *7*. [c] Extrapolated from measured values by means of the Arrhenius equation. [d] Calculated from data reported in ref *7*.

will lead to a faster reaction rate. We have thus experimentally determined the stability of the carbanion intermediates using mass spectrometry. It would be very interesting to study the carbanion that would result from decarboxylation of acid **7**; however, its carboxylate and its decarboxylation product are neutral, so mass spectrometry studies are not possible. It is important to point out that the order of stability for the carbanions determined in the gas phase should be the same as that in solution due to their similarity in structure and thus in solvation. Furthermore, if the active site of ODCase is hydrophobic (vide infra), gas-phase studies will provide a useful model for the active site environment (i.e., low dielectric medium).

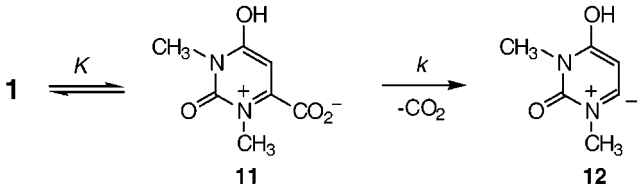
The carbanions **8**, **9**, and **10** were formed in a quadrupole ion trap mass spectrometer via collision-activated dissociation (CAD) of precursor carboxylate anions derived from **1**, **5**, and **6** (*17*). The carboxylates were generated by electrospray ionization (ESI). The proton affinities of carbanions **8**, **9**, and **10** were measured by allowing them to react with a series of neutral acids of varying strength and bracketing them between the weakest acid that gave a proton transfer and

Table 2: Experimental and Calculated Proton Affinities

carbanions	calculated ΔH (kcal/mol)[a]	experimental ΔH (kcal/mol)
8	367.6 (366.0)	369.9 ± 3.1
9	375.5 (374.0)	377.0 ± 2.9
10	375.8 (375.0)	377.0 ± 2.9

[a] Values calculated at the MP2/6-31+G(d,p)//HF/6-31+G(d) level. B3LYP/6-31+G(d,p)//HF/6-31+G(d) values are given parenthetically.

Scheme 4



$dP/dt = Kk[1]/(K+1)$ When $K << 1$, $k_{ob} = Kk$

the strongest that did not (*17*). To support the experimental work, ab initio calculations were carried out at the MP2/6-31+G(d,p)//HF/6-31+G(d) and B3LYP/6-31+G(d,p)//HF/6-31+G(d) levels to determine the proton affinities of the carbanions (*17*). The experimental and theoretical values are in good agreement, and the results are shown in Table 2. It should be pointed out that the experiments are indeed probing the proton affinities of the carbanions with negative charges at the desired sites (as depicted in structures **8**−**10**) since theoretical calculations have shown that carbanions **8**−**10** are much more stable than isomeric carbanions with the negative charge on other carbons (*17*). Theoretical calculations have further suggested that the activation barrier for decarboxylation in the gas phase is the same as the endothermicity of decarboxylation; i.e., there is no barrier to the reverse reaction, the addition of CO_2 to the carbanion (*18*).

Carbanion **8** from the decarboxylation of 1,3-dimethylorotic acid (**1**) is unusually stable, and its proton affinity in the gas phase is comparable to that of an enolate. The data also indicate that carbanions **9** and **10** are about equally stable and are about 7 kcal/mol less stable than carbanion **8**. However, as shown in Table 1, the rate constant for decarboxylation of acid **6** is about 10^3-fold greater than that of either **1** or **5**. These observations suggest that the stability of the carbanion intermediate does not play a major role in determining the rate of decarboxylation.

We then set out to find an alternative explanation for the rate differences among acids **1**, **5**, and **6** since the stability of the carbanions does not appear to be the controlling factor. Beak and Siegel proposed the zwitterion mechanism for the decarboxylation of 1,3-dimethylorotic acid as shown in Scheme 4 (*7*). The reaction mechanism involves initial formation of zwitterion **11** and subsequent loss of CO_2 to form carbanion intermediate **12** (*7*).

Kinetic analysis of the above reaction scheme is also shown in Scheme 4 (*7*). The observed rate constant k_{ob} is composed of *K*, the equilibrium constant for the formation of zwitterion **11**, and *k*, the rate constant for the loss of CO_2 leading to carbanion **12**. Under the reaction conditions, *K* should be much smaller than 1 for all the analogues discussed here, and k_{ob} is thus simply the product of *K* and *k*. We hypothesize that *k*'s for all the analogues are roughly similar

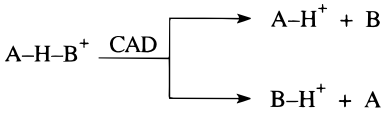
Table 3: Experimental and Calculated Proton Affinities of Orotic Acid Analogues

acid	calculated proton affinity[a,b] (kcal/mol)	experimental proton affinity[a] (kcal/mol)
1	209.6 (211.5)	210.2 ± 1.4
5	218.7 (217.8)	218.7 ± 2.7
6	231.5 (230.3)	232.5 ± 3.2

[a] The proton affinities are for the most basic site in each case. In **1**, this is oxygen-4. [b] Values calculated at the MP2/6-31+G(d,p)//HF/6-31+G(d) level. B3LYP/6-31+G(d,p)//HF/6-31+G(d) values are given parenthetically.

and that the equilibrium constant for the formation of the zwitterion, *K*, largely determines the overall reaction rate.

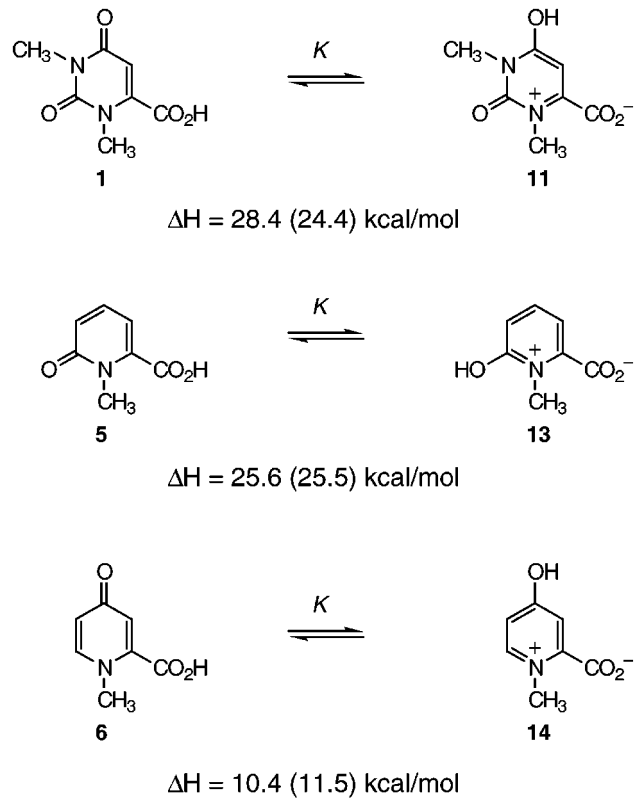
To test this hypothesis, we need to compare the proton affinities or the basicity of the corresponding oxygens in the orotic acid analogues **1**, **5**, and **6**. Again, mass spectrometry was employed to determine the proton affinities. In this case, Cooks' kinetic method was used to measure the proton affinities (*17*, *19*). This method has emerged as a rapid and precise method of determining proton affinities and involves forming a proton-bound complex of the substrate (A) and a reference base (B). Collision-activated dissociation (CAD) of the complex generally leads to two ionic products (the protonated substrate and reference), and the ratio of the products can be quantitatively related to the relative proton affinities.



The experimental proton affinities of these acids are shown in Table 3. The experimental data are in good agreement with theoretical calculations at the MP2/6-31+G(d,p)//HF/6-31+G(d) and B3LYP/6-31+G(d,p)//HF/6-31+G(d) levels, also shown in Table 3. The calculations indicate that the proton affinities for the carboxylic acid oxygens are low so there is little doubt that we are measuring the proton affinities of the ring carbonyl oxygens in the experiments. These values suggest that the oxygen in acid **6** is much more basic than the oxygens in acids **1** and **5**. This order of basicity is in agreement with that of uracil, 2-pyridone, and 4-pyridone in aqueous solution, where pK_a values of 0.6 (*20*), 0.70 (*21*) [1.25 in another paper (*22*)], and 3.27 (*20*, *21*) have been reported, respectively.

The results on the basicity of the oxygens seem to correlate with the relative rate constants of the corresponding acids. The more basic the oxygen, the higher the fraction of the zwitterion structure in the substrate solution. However, our hypothesis suggests that the most important factor is the equilibrium constants, *K*, between the orotic acid analogues and their zwitterion structures as shown in Scheme 5. Unfortunately, the energy differences between these structures cannot be determined experimentally. Our results have consistently demonstrated that the calculated values are in excellent agreement with the experimental values. We have thus calculated the energy differences among these structures at the MP2/6-31+G(d,p)//HF/6-31+G(d) and B3LYP/6-31+G(d,p)//HF/6-31+G(d) levels, with the latter values shown in parentheses in Scheme 5. Comparison of the energy differences between acids **1**, **5**, and **6** and their zwitterions

Scheme 5



shows that zwitterion **14** from acid **6** is much more favored than its counterparts **11** and **13**. Furthermore, the equilibria between **1** and **11** as well as **5** and **13**, respectively, are about equally positioned. Kinetic results in Table 1 have demonstrated that acid **6** decarboxylates much faster than either acid **1** or acid **5**, which have nearly the same rate constants.

The excellent correlation between the above results on the equilibrium leading to the formation of the zwitterion structures and rate constants of decarboxylation of acids **1**, **5**, and **6** provides strong support for our hypothesis that the equilibrium constant *K* for the formation of the zwitterion plays a dominant role in the rate of decarboxylation. This hypothesis would thus argue that the enzymatic decarboxylation of OMP would be greatly accelerated if the substrate is bound in the zwitterion form in the active site of ODCase. The rate constant for the decarboxylation of zwitterion **2** can be roughly estimated from that of betaine **7** (*7*). As can be seen in Table 1, betaine **7** decarboxylates about 10^8-fold faster than acid **1**, which suggests that the decarboxylation of OMP can be accelerated by a factor of 10^8 if it is bound in the zwitterion form (structure **2** or its O-4 protonated counterpart or the double-protonated form) in the enzyme active site. Comparison of the rate of decarboxylation of betaine **7** at 25 °C (Table 1) with that reported for 1-methylorotic acid (*6*) reveals an even more impressive enhancement of 5×10^9-fold.

It is thus very likely that one way in which ODCase catalyzes the decarboxylation of OMP is the binding of the substrate in the energetically unfavorable zwitterion form as proposed by Beak and Siegel (*7*). This is an interesting example of enzymatic catalysis by binding the substrate in an altered state as described by the strain or distortion theory elegantly discussed by Jencks (*23*). The strain theory suggests that the binding energy between the substrate and the enzyme is directly utilized to induce strain and to catalyze the reaction. Although it is often very hard to draw the line between stabilization of the transition state and destabilization of substrate, the latter scenario may be more likely in this case since the zwitterion form of the substrate could exist as a discrete intermediate. The relatively rigid and highly functionalized heterocycles and phosphoribose components of nucleotides may enjoy a special advantage in that the entropic costs of binding can be greatly reduced, as suggested by Carlow et al. in their study on cytidine deaminase (*24*). They have observed that binding to the 3′-hydroxy of the ribose moiety, which is far away from the reaction site, contributes as much as 5.2 kcal/mol in free energy in the reduction of reaction activation energy. In ODCase, binding energy to remote parts of OMP, such as the phosphoribose, can be used to force the substrate into the zwitterion form and thus facilitate decarboxylation.

Although zwitterion formation seems to be a very important factor in the catalysis, it only accounts for about 10^8−10^{10}-fold of the 10^{17}-fold rate enhancement seen in enzyme-catalyzed decarboxylation. On the other hand, it seems unlikely that the enzyme can substantially stabilize the ylide intermediate any further without electron sink cofactors. Moreover, as discussed above, the stability of the carbanion may only have a limited effect on the rate of decarboxylation. This poses an interesting question as to how ODCase accelerates the decarboxylation after the substrate OMP is bound in the zwitterion form. This question is not unique to ODCase. In decarboxylases that utilize PLP or TPP as cofactors, the rate acceleration observed in the enzymatic reaction cannot be fully accounted for by the chemical stabilization of carbanion intermediates. As noted by Marlier and O'Leary, the rates of enzymatic decarboxylations using either PLP or TPP as cofactors exceed those of model compounds covalently bonded to PLP or TPP analogues by many orders of magnitude (*25*).

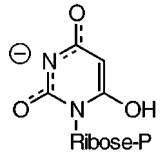
Decarboxylation could be further accelerated by destabilization of the substrate carboxylate group in the active site of ODCase, since additional stabilization of the intermediate is not very likely and may have a very limited impact. It can be envisioned that destabilization of carboxylate groups may be accomplished by two possible ways: a hydrophobic environment or negative charges nearby. Both phenomena have been observed in biological catalytic systems. O'Leary and Piazza pointed out that medium effects and desolvation might play a role in enzymatic catalysis of decarboxylation (*26*). Recently Jordan et al. have reported that the low-polarity environment of the active site of TPP-dependent pyruvate decarboxylase could account for a billion-fold rate acceleration (*27*). There are also some examples of enzymes and catalytic antibodies that contain a hydrophobic binding pocket to desolvate the carboxylate group (*28*−*30*). Moreover, a monoclonal antibody elicited against a hydrophobic hapten has been found to catalyze the decarboxylation of OMP by a factor of 10^8 (*31*). On the other hand, histidine decarboxylase binds the carboxylate group in a hydrophobic pocket that also contains a glutamate residue, presumably to further destabilize the substrate carboxylate group (*28*). It is conceptually obvious that negative charges in the close vicinity of the carboxylate group will destabilize it to facilitate decarboxylation. Unfortunately, it is very hard to

develop model studies to test for a rate enhancement by nearby negative charges.

On the contrary, the effect of a hydrophobic medium on the rate of decarboxylation has been extensively documented in model studies. For example, the rate of decarboxylation for 4-pyridylacetic acid and the rate of a model reaction for the TPP-dependent decarboxylation increase substantially in media of decreasing polarity (*25*, *32*, *33*). Similar results have been reported by Kemp et al. in the decarboxylation of benzisoxazole-3-carboxylic acid, in which the rate of decarboxylation increases by 10^8-fold from aqueous solution to organic solution (*34*). It has been suggested that this phenomenal acceleration in reaction rate is due to the desolvation of the carboxylate ion in organic solvents (*35*). In our studies on the decarboxylation of orotic acid analogues in a mass spectrometer, we have observed that these analogues decarboxylate very efficiently in the electrospray source. Although the carboxylate anions are not entirely desolvated in the heated capillary of the ESI source (desolvation is occurring down the length of the capillary), the gas phase half-life for the conversion of the carboxylate anion of acid **1** to carbanion **8** is on the order of a millisecond when the capillary walls are heated to 200 °C. This observation suggests a rate constant of approximately 10^3 s^{-1} for the decarboxylation under these conditions. By comparison with the reaction rates in solution as shown in Table 1, it is apparent that a desolvating environment is capable of greatly accelerating the decarboxylation reaction; however, the possibility of energetic collisions in the heated capillary region [i.e., collision-activated dissociation (CAD)] precludes any quantitative comparison. It should be noted that in these experiments, the electrostatic lenses in the instrument were adjusted to minimize the amount of CAD in the electrospray source.

The combination of the accelerating effect from zwitterion formation and destabilization of the carboxylate group could adequately account for the 10^{17}-fold rate enhancement seen in ODCase. We would thus like to propose a new mechanism for the decarboxylation of OMP by ODCase in which the enzyme uses its strong binding with the polyfunctional and charged phosphoribose moiety of the substrate to favor the formation of a zwitterion intermediate such as **2** or an analogous structure and to force the negatively charged carboxylate group into a destabilizing environment such as a desolvating hydrophobic binding pocket, an electrostatically repulsive pocket, or a combination of both as seen in histidine decarboxylase (*28*). Therefore, the enzyme catalyzes the decarboxylation of OMP via destabilization of the substrate, and not by the stabilization of the carbanion intermediate/transition state. The formation of a zwitterion intermediate prior to decarboxylation is consistent with recent studies on kinetic isotope effects, which suggest protonation followed by decarboxylation in contrast to the concerted carbene mechanism (*36*).

The formation of a zwitterion intermediate required by this mechanism can explain the extraordinarily potent binding ($K_i = 9 \times 10^{-12}$ M) between 1-β-D-ribofuranosylbarbiturate 5′-monophosphate (BMP, one of its resonance forms is shown below as structure **15**) and ODCase (*14*). The pyrimidine ring in BMP is quite acidic ($pK_a = \sim 4.5$) and is negatively charged at physiological pH whereas the pyrimidine rings in OMP and UMP are not (*14*). The extremely tight binding between BMP and ODCase may be the result of the strong interaction between the negatively charged exocyclic oxygen of BMP and positively charged residues in the active site such as lysine 93 (*37*). This may lead to rational design of UMP analogues with a negatively charged phenolic oxygen as potent inhibitors of ODCase. Considering the important biological role of this enzyme, these inhibitors could be of potential medicinal uses.



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Model studies in our laboratories have demonstrated that the stability of carbanion intermediates has only a very limited effect on the rate of decarboxylation for orotic acid analogues. On the other hand, the position of the equilibrium between the neutral acids and the zwitterions (i.e., the population of zwitterions in solution) plays a major role in determining the rate of decarboxylation. We have thus proposed that ODCase catalyzes the decarboxylation of OMP through destabilization of the substrate, instead of stabilization of the intermediate/transition state. Furthermore, destabilization of the substrate is accomplished in two ways: binding the substrate in the normally much less stable zwitterionic form and placing the carboxylate group in a destabilizing environment such as a desolvating hydrophobic binding pocket, an electrostatically repulsive pocket, or a combination of both. We further believe that this kind of destabilization of substrate is made possible by the tremendous binding energy from the interaction between ODCase and the polyfunctional nucleotide substrate, especially the phosphoribose moiety. It is likely that most of the binding occurs at the phosphoribose segment (*24*). We are currently probing the enzyme active site environment both structurally and chemically, investigating the structure of the substrate when bound to ODCase, and synthesizing analogues as inhibitors or alternative substrates.

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