Theoretical Studies of Mechanisms and Kinetic Isotope Effects on the Decarboxylation of Orotic Acid and Derivatives

Linda M. Phillips and Jeehiun K. Lee*

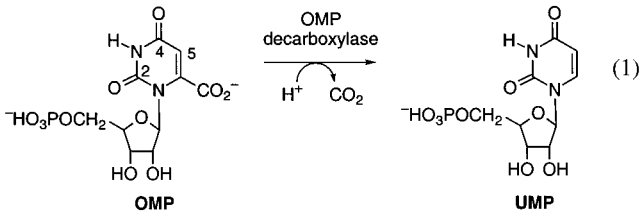
Contribution from the Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854

Received July 16, 2001

Abstract: The mechanism of orotidine 5′-monophosphate decarboxylase was studied computationally by using the decarboxylation of orotic acid analogues as model systems. These calculations indicate that mechanisms involving proton transfer to the 2-oxygen or the 4-oxygen are energetically favorable, as compared to direct decarboxylation without proton transfer, for a series of model compounds where N1 is substituted with respectively H, CH_3, and a tetrahydrofuran moiety. Proton transfer to the 4-oxygen during decarboxylation is found to be energetically more favorable than 2-protonation, which is attributable to both the 4-oxygen site being more basic and an apparent intrinsic preference for the 4-protonation pathway. ^{15}N isotope effect calculations were also conducted, and compared to experimental ^{15}N isotope effects previously measured at N1 by Rishavy and Cleland (*Biochemistry* **2000**, *39*, 4569−4574). The theoretical isotope effects establish, for the first time, that the experimental ^{15}N isotope effect is consistent with decarboxylation *without* protonation, as well as with decarboxylation *with* protonation, at either O2 or at O4. Furthermore, we propose herein an isotope measurement that could potentially distinguish among mechanisms involving protonation from those that do not involve proton transfer.

Introduction

OMP decarboxylase (ODCase) is a key enzyme in the biosynthesis of nucleic acids, effecting the decarboxylation of orotidine 5′-monophosphate (OMP) to form uridine 5′-monophosphate (UMP, eq 1).[1,2] This conversion of OMP to UMP is



(1)

of biomechanistic interest, because the decarboxylation results, uniquely, in a carbanion that cannot delocalize into a π orbital.[3,4] The uncatalyzed reaction is therefore extremely unfavorable, and ODCase is one of the most proficient enzymes known, with a $k_{cat}/K_m/k_{non}$ of 2.0×10^{23} M^{-1}.[1,5]

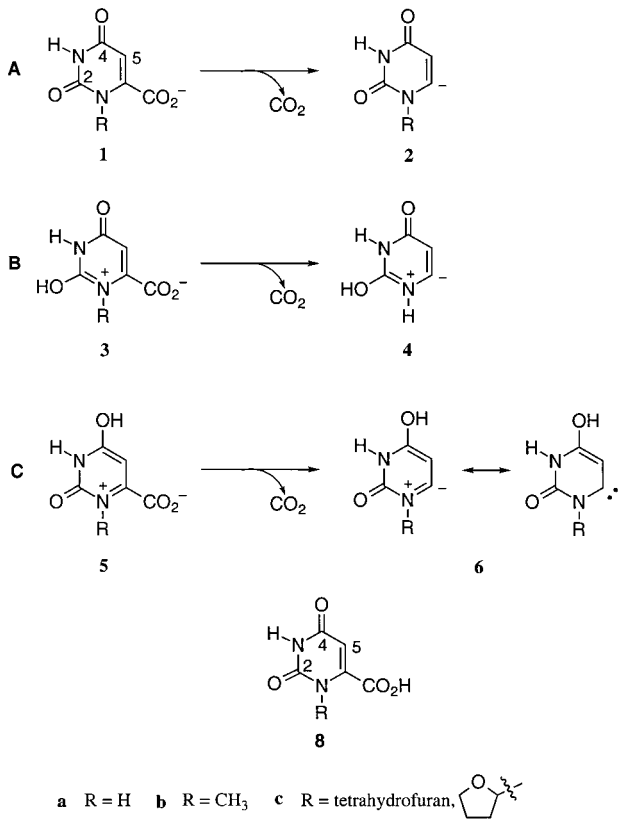
Because of its essential role in nucleic acid biosynthesis, and because of its unique mechanistic characteristics, ODCase has long been the subject of much study, but the catalytic mechanism remains unknown.[6−17] Studies of the enzyme mechanism by Jones and Smiley indicate that Lys93 (in the yeast enzyme) is important for catalysis, but not for binding.[18]

Various mechanistic hypotheses have been proposed to explain the fantastic catalysis by ODCase. Prevalent among them is proton transfer to the 2-oxygen (the "ylide" mechanism) or to the 4-oxygen (the "carbene" mechanism), proposed by Beak and co-workers, and Lee and Houk, respectively (Scheme 1, reactions B and C).[6,19] Lee and Houk also proposed that the intermediate formed upon 4-protonation and decarboxylation (**6**) could be stabilized as reflected in the carbene resonance structure.[20,21] More recently, and very importantly, four different crystal structures of ODCase have been solved and reported,

(1) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(2) McClard, R. W.; Black, M. J.; Livingstone, L. R.; Jones, M. E. *Biochemistry* **1980**, *19*, 4699−4706.
(3) Bruice, T. C.; Benkovic, S. *Bioorganic Mechanisms*; W. A. Benjamin: New York, 1966; Vol. 2.
(4) Bender, M. L. *Mechanisms of Homogeneous Catalysis from Protons to Proteins*; Wiley-Interscience: New York, 1971.
(5) Snider, M. J.; Wolfenden, R. *J. Am. Chem. Soc.* **2000**, *122*, 11507−11508.
(6) Beak, P.; Siegel, B. *J. Am. Chem. Soc.* **1976**, *98*, 3601−3606.
(7) Acheson, S. A.; Bell, J. B.; Jones, M. E.; Wolfenden, R. *Biochemistry* **1990**, *29*, 3198−3202.
(8) Smiley, J. A.; Paneth, P.; O'Leary, M. H.; Bell, J. B.; Jones, M. E. *Biochemistry* **1991**, *30*, 6216−6223.
(9) Smiley, J. A.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8319−8323.
(10) Levine, H. L.; Brody, R. S.; Westheimer, F. H. *Biochemistry* **1980**, *19*, 4993−4999.
(11) Smiley, J. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 3877−3878.
(12) Silverman, R. B.; Groziak, M. P. *J. Am. Chem. Soc.* **1982**, *104*, 6434−6439.
(13) Cui, W.; DeWitt, J. G.; Miller, S. M.; Wu, W. *Biochem. Biophys. Res. Commun.* **1999**, *259*, 133−135.
(14) Ehrlich, J. I.; Hwang, C.-C.; Cook, P. F.; Blanchard, J. S. *J. Am. Chem. Soc.* **1999**, *121*, 6966−6967.
(15) Nakanishi, M. P.; Wu, W. *Tetrahedron Lett.* **1998**, *39*, 6271−6272.
(16) Wu, W.; Ley-han, A.; Wong, F. M.; Austin, T. J.; Miller, S. M. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2623−2628.
(17) Singleton, D. A.; Merrigan, S. A.; Kim, B. J.; Beak, P.; Phillips, L. M.; Lee, J. K. *J. Am. Chem. Soc.* **2000**, *122*, 3296−3300.
(18) Smiley, J. A.; Jones, M. E. *Biochemistry* **1992**, *31*, 12162−12168.
(19) Lee, J. K.; Houk, K. N. *Science* **1997**, *276*, 942−945.
(20) Lavorato, D.; Terlouw, J. K.; Dargel, T. K.; Koch, W.; McGibbon, G. A.; Schwarz, H. *J. Am. Chem. Soc.* **1996**, *118*, 11898−11904 and references therein.
(21) Arduengo, A. J. I.; Dias, H. V. R.; Dixon, D. A.; Harlow, R. L.; Klooster, W. T.; Koetzle, T. F. *J. Am. Chem. Soc.* **1994**, *116*, 6812−6822 and references therein.

10.1021/ja0117332 CCC: $20.00
Published on Web 11/09/2001

Scheme 1



by the groups of Ealick and Begley,[22] Short and Wolfenden,[23] Larsen,[24] and Pai and Gao.[25−29] Examination of these crystal structures gave rise to a third mechanistic proposal, involving a direct decarboxylation without proton transfer, where catalysis is achieved through ground-state destabilization. The ground-state destabilization hypothesis has led to additional studies probing this mechanistic hypothesis, as well as much debate, including isotope effect studies by Cleland et al., described in more detail later in this paper, that appear consistent with a direct decarboxylation mechanism, where no proton transfer is involved.[23,26−36]

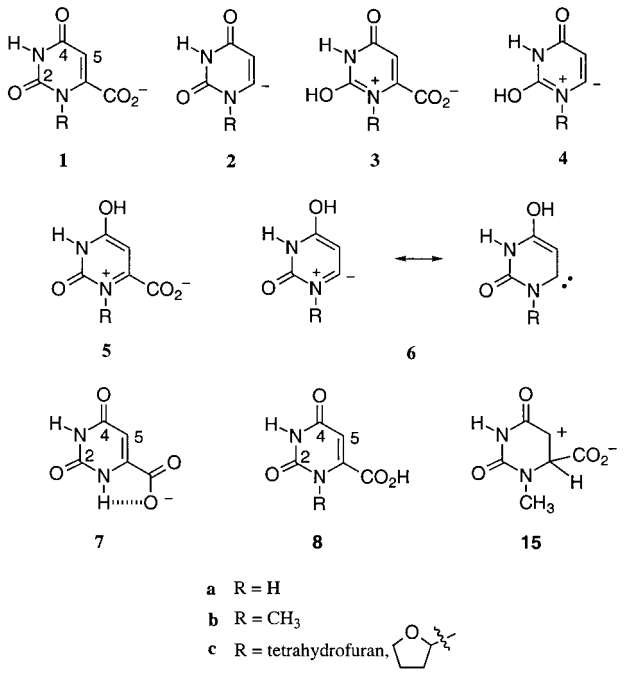
An enzyme as proficient as ODCase is expected to be unusually "sensitive to ... reversible inhibitors [that are] designed to resemble the [transition structure]".[1] The biological and medicinal importance of this fact is clear: as a key biosynthetic step, the decarboxylation is a natural target for antitumor agents and genetic diseases such as orotic aciduria. Knowledge of the transition structure facilitates inhibitor design; therefore, understanding the mechanism is paramount in controlling it. We describe here a theoretical study of three possible catalytic mechanisms of decarboxylation: direct decarboxylation (no proton transfer, Scheme 1, reaction A), the ylide mechanism (proton transfer to the 2-oxygen with decarboxylation, Scheme 1, reaction B), and the carbene mechanism (proton transfer to the 4-oxygen with decarboxylation, Scheme 1, reaction C). We show that energetically, protonation is a viable means of catalysis, particularly proton transfer to the 4-oxygen, for a series of orotic acid derivatives. Isotope effect calculations confirm for the first time that the previously measured ^{15}N isotope effect for the N1 of OMP in ODCase is consistent with decarboxylation without protonation, as well as with mechanisms involving proton transfer.[36] The calculations indicate that the ^{15}N isotope effect at N1 cannot differentiate among the three possible mechanisms, and we propose an isotope measurement that could potentially differentiate among these mechanistic possibilities.

Theoretical Methods

The geometries of all structures described in the text were optimized at RHF/6-31+G*. MP2/6-31+G* single points were conducted on the RHF geometries; final energetics reported are at MP2/6-31+G*//RHF/6-31+G*, and do not include zero-point energies, as the tetrahydrofuran (THF) moiety made frequency calculations prohibitively expensive. The MP2/6-31+G*//RHF/6-31+G* level has been shown previously to give reliable values for orotate decarboxylation.[19] Where R = THF, the starting structure used for the calculations was the crystal structure for uridine 5′-monophosphate bound to ODCase in *Bacillus subtilis*.[22,30] We then also conducted calculations for a series of structures resulting from rotation about the N1−C1′ nucleobase−sugar bond. The C2−N1−C1′−O′ dihedral angle in the crystal structure is 69°; we conducted partial optimizations, changing the dihedral by successive 60° increments. We found that for all the reactants, the energetically preferred dihedral angle is 69°, while for the products, the preferred dihedral angle is −111°. We used the preferred partially optimized structures as the starting points for full optimizations to obtain the final barriers. Structures **1c**, **3c**, and **5c** optimized with C2−N1−C1′−O′ dihedral angles of 53°, 44°, and 53°, respectively; structures **2c**, **4c**, and **6c** optimized with dihedral angles of −109°, −105°, and −114°, respectively. For structures **1**−**6**, where R = H and methyl, we also conducted B3LYP/6-311++G calculations to benchmark the MP2 single points. B3LYP methods have also been previously shown to provide reliable relative energetics for decarboxylations.[17,19,37,38] Gaussian94 and Gaussian98 were used for all the computations.[39,40]

For the isotope effects, frequency calculations were conducted on the B3LYP/6-311++G optimized structures and isotope effects were theoretically determined by using the program Quiver.[41−43] A scaling factor of 0.96 was used.[44]

(22) Appleby, T. C.; Kinsland, C.; Begley, T. P.; Ealick, S. E. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2005−2010.
(23) Miller, B. G.; Hassell, A. M.; Wolfenden, R.; Milburn, M. V.; Short, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2011−2016.
(24) Harris, P.; Poulsen, J.-C. N.; Jensen, K. F.; Larsen, S. *Biochemistry* **2000**, *39*, 4217−4224.
(25) Wu, N.; Mo, Y.; Gao, J.; Pai, E. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2017−2022.
(26) Houk, K. N.; Lee, J. K.; Tantillo, D. J.; Bahmanyar, S.; Hietbrink, B. N. *ChemBioChem* **2001**, *2*, 113−118.
(27) Warshel, A.; Florian, J.; Strajbl, M.; Villa, J. *ChemBioChem* **2001**, *2*, 109−111.
(28) Warshel, A.; Florian, J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5950−5955, and references therein.
(29) Warshel, A.; Strajbl, M.; Villa, J.; Florian, J. *Biochemistry* **2000**, *39*, 14728−14738.
(30) Begley, T. P.; Appleby, T. C.; Ealick, S. E. *Curr. Opin. Struct. Biol.* **2000**, *10*, 711−718.
(31) Miller, B. G.; Butterfoss, G. L.; Short, S. A.; Wolfenden, R. *Biochemistry* **2001**, *40*, 6227−6232.
(32) Miller, B. G.; Snider, M. J.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 8113−8118.
(33) Miller, B. G.; Snider, M. J.; Wolfenden, R.; Short, S. A. *J. Biol. Chem.* **2001**, *276*, 15174−15176.
(34) Miller, B. G.; Traut, T. W.; Wolfenden, R. *Bioorg. Chem.* **1998**, *26*, 283−288.
(35) Smiley, J. A.; Hay, K. M.; Levison, B. S. *Bioorg. Chem.* **2001**, *29*, 96−106.
(36) Rishavy, M. A.; Cleland, W. W. *Biochemistry* **2000**, *39*, 4569−4574.
(37) Bach, R. D.; Canepa, C.; Glukhovtsev, M. N. *J. Am. Chem. Soc.* **1999**, *119*, 6542−6555.
(38) Bach, R. D.; Canepa, C. *J. Am. Chem. Soc.* **1997**, *119*, 11725−11733.
(39) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. *GAUSSIAN94*, Revision E.2; Gaussian, Inc.: Pittsburgh, PA, 1995.



Results and Discussion

Energetics Calculations. The energetics of the decarboxylation for 1-methyl orotate (**1b**) to form vinylic carbanion **2b** (Scheme 1, reaction A), at MP2/6-31+G*//RHF/6-31+G*, are summarized in Table 1 (column 2, nonparenthetical values).[17] The reaction of **1b** is endothermic in the gas phase by a significant amount and there is no barrier to recombination of CO_2 with the carbanion, such that the forward reaction leads smoothly to a flat plateau, and endothermicity and the barrier are essentially the same (activation energy, $\Delta E^{\ddagger} = 41.3$ kcal mol^{-1}). Protonation on the 2-oxygen (**3b** → **4b**) significantly lowers the barrier, to 16.9 kcal mol^{-1}. Protonation on the 4-oxygen (**5b**), followed by decarboxylation to form the carbene (**6b**) proposed by Lee and Houk, is slightly more favorable, with a barrier of 15.1 kcal mol^{-1}. Therefore, energetically speaking, protonation is a viable mechanism for significantly lowering the barrier to decarboxylation; the enzyme lowers the barrier by 23 kcal mol^{-1}, and protonation on the 2- or the 4-oxygen could definitely yield such a barrier lowering.[1] Calculations on the 1-methyl orotate system have also been conducted previously at B3LYP/6-31+G*, and are consistent with our results.[17]

We expected protonation to lower the barrier; however, the results of the relative energetics of the 2- versus the 4-protonated species were surprising to us. Earlier calculations by Lee and Houk on the parent orotates (R = H; Table 1, first column) also indicated that 4-protonation was favored, but that the energetic preference for the decarboxylation of **5a** versus **3a** was much greater; that is, 4-protonation/decarboxylation was calculated to be 6.3 kcal mol^{-1} more favorable than 2-protonation/decarboxylation.[17,19] We find a preference of about 2 kcal mol^{-1}. Furthermore, the absolute values for the barriers for the three substrates **1**, **3**, and **5** are significantly larger for the R = H species as compared to the R = CH_3 species. For the uncatalyzed decarboxylation, the R = H system has a barrier of 47.6 kcal mol^{-1}, 6.3 kcal mol^{-1} higher than when R = CH_3. For 2-protonation, the barrier is higher in the R = H system by 8.2 kcal mol^{-1}. For 4-protonation, the barrier difference is 3.6 kcal mol^{-1}.

We believe that the phenomenon responsible for this change in relative energetics is simply an internal hydrogen bond between the proton on N1 and one of the carboxylate oxygens (**7**).[17] Figure 1 shows the calculated geometries for **1a**, **3a**, and **5a**. The distance between the N1 proton and the carboxylate oxygen is 2.136 Å for the unprotonated substrate **1a**, 1.971 Å for 2-protonated substrate **3a**, and 2.124 Å for 4-protonated substrate **5a**. This internal hydrogen bond stabilizes the ground state, and does so significantly for the 2-protonated substrate **3a**, which has the shortest—and therefore the most stable—internal hydrogen bond. This unusual stabilization of the ground state **3a** creates an unexpectedly large calculated barrier for the decarboxylation, such that it appears disfavored as compared to the decarboxylation of **5a**, by 6 kcal mol^{-1}.

Replacement of the proton on N1 with a methyl group eliminates the possibility of the internal hydrogen bond, and the energetics reflect that change.[17]

As a benchmark for the MP2 computations, we also calculated energetics at B3LYP/6-311++G (Table 1, parenthetical values); although the values are slightly higher than at the MP2 level, the relative energetics are consistent.

Because of the difference between the orotate and 1-methyl orotate systems, we decided to examine a more realistic model of the parent OMP system, and explored the energetics where R = tetrahydrofuran (THF; structures **1**−**6c**) to mimic the ribose moiety on the true OMP substrate; energetics are summarized in Table 1 (last column).

Table 1. $\Delta E^{\ddagger}$ for the Decarboxylation of Substrates **1**, **3**, and **5** at the MP2/6-31+G*//RHF/6-31+G* Level (kcal mol^{-1})[a]

reaction	R = H[b]	R = CH_3	R = tetrahydrofuran
1 → **2** + CO_2	47.6 *(54.3)*	41.3 *(46.6)*	39.3
3 → **4** + CO_2	25.1 *(31.4)*	16.9 *(21.8)*	17.0
5 → **6** + CO_2	18.7 *(24.5)*	15.1 *(20.8)*	15.4

[a] B3LYP/6-311++G values are in parentheses. [b] Reference 19.

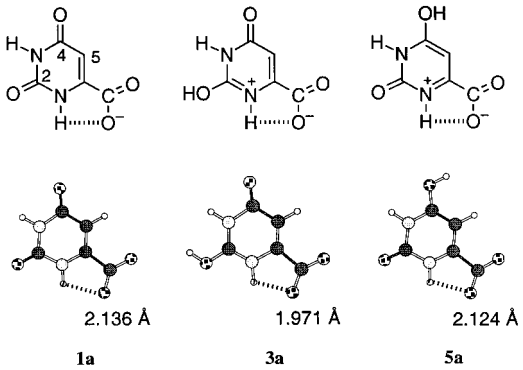


Figure 1. Optimized structures (RHF/6-31+G*) for each of the reactants **1a**, **3a**, and **5a**. The length of each hydrogen bond between the N1 proton and the carboxylate oxygen is indicated in Å.

(40) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Baboul, A. G.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andres, J. L.; Gonzalez, C.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *GAUSSIAN98*; Gaussian, Inc.: Pittsburgh, PA, 1998.

(41) Bigeleisen, J.; Mayer, M. G. *J. Chem. Phys.* **1947**, *15*, 261.

(42) Wolfsberg, M. *Acc. Chem. Res.* **1972**, *5*, 225−233.

(43) Saunders, M.; Laidig, K. E.; Wolfsberg, M. *J. Am. Chem. Soc.* **1989**, *111*, 8989−8994.

(44) Scott, A. P.; Radom, L. *J. Phys. Chem.* **1996**, *100*, 16502−16513.

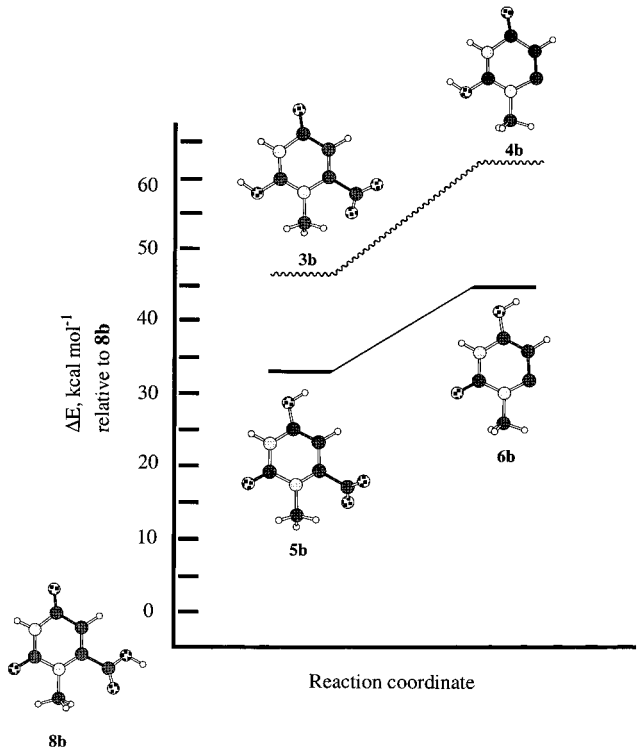


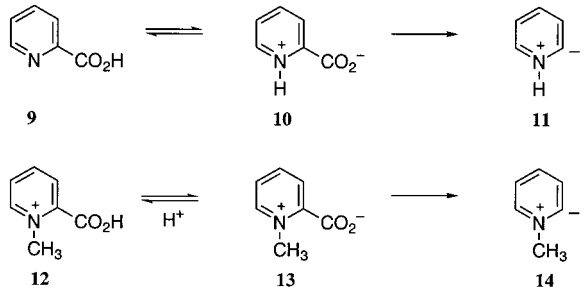
Figure 2. Calculated $\Delta E^{\ddagger}$ values (MP2/6-31+G*//RHF/6-31+G*) for the decarboxylation of **3b** → **4b (+ CO_2)** and **5b** → **6b (+ CO_2)**, relative to **8b**.

When there is no protonation, the parent substrate decarboxylates with a large barrier of 39.3 kcal mol^{-1}. 2-Protonation lowers the barrier to 17.0 kcal mol^{-1}, while 4-protonation lowers the barrier to 15.4 kcal mol^{-1}. These data are consistent with the R = Me cases, and again, a slight but real preference for the 4-protonation path to form the carbene is observed.

In addition to the intrinsic preference for the 4-protonation/carbene mechanism (**5** → **6**) over the 2-protonation/ylide mechanism (**3** → **4**) is the greater basicity of the 4 position, which means that the overall barrier to form **6** versus **4** from a common reference point such as the orotic acid **8** is *substantially* lower for 4-protonation (Figure 2).[17] Our calculations show that for the decarboxylation of the 2-protonated substrate **3b**, relative to **8b**, the barrier is 63.2 kcal mol^{-1}, whereas for the 4-protonated substrate **5b**, the barrier is 45.6 kcal mol^{-1}. Comparable values are found for R = THF: 2-protonation, 61.9 kcal mol^{-1}; 4-protonation, 46.1 kcal mol^{-1}. Therefore, in summary, the carbene mechanism (decarboxylation with protonation at O4) is the energetically favored pathway. Our calculations show that this preference is a result of both the 4 position being more basic and a slight intrinsic preference for that pathway. Previous experimental results are in agreement with these computational predictions; isotope effect studies show that 1,3-dimethyl orotic acid decarboxylates in sulfolane via 4-protonation.[17] We predict that the R = THF model compounds will behave similarly in solution.

Kinetic Isotope Effects. In an effort to probe further the viability of proton transfer as a mechanism for catalysis, we conducted ^{15}N isotope effect (IE) studies on the N1 position of 1-methyl orotate. Recently, Cleland and co-workers measured the ^{15}N isotope effect at N1 for the decarboxylation of OMP in ODCase, as well as of picolinic acid and *N*-methyl picolinic acid (Table 2, Scheme 2).[36,45] Because decarboxylation is the rate-determining step, any observed isotope effect should represent the isotope effect associated with the decarboxylation

Scheme 2



step multiplied by the isotope effect for formation of any intermediate prior to the decarboxylation. The authors therefore propose that should ODCase effect catalysis through, for example, protonation on the 2-oxygen to form the zwitterion **4** (the ylide mechanism) prior to decarboxylation, a large, inverse isotope effect due to the "formation of a quaternary nitrogen intermediate will contribute to the observed ^{15}N effect".[36] Conversely, if no intermediate is formed prior to decarboxylation, the N1 should presumably remain ternary throughout the reaction, and a normal IE should be observed.

For example, the protonation of pyridine, in which the nitrogen changes from ternary to quaternary, has an equilibrium ^{15}N IE of 0.9793.[46] To provide further evidence for "bond order changes" at N1 influencing the overall IE, the authors also measured the ^{15}N−N1 IE's for the decarboxylation of picolinic acid and for *N*-methyl picolinic acid.

For picolinic acid (**9**, Scheme 2) to decarboxylate, a zwitterion must presumably be formed by proton transfer from the carboxylic acid to the nitrogen. The protonation of the nitrogen should contribute an inverse ^{15}N isotope effect at N1, and indeed, an isotope effect for the decarboxylation of 0.9955 at 463 K (Table 2, corrects to 0.9930 at 298 K) is measured. *N*-Methyl picolinic acid, on the contrary (**12**, Scheme 2), should yield a normal ^{15}N−N1 IE for decarboxylation. The nitrogen begins the reaction quaternary and remains quaternary throughout the reaction; the authors do in fact measure a normal IE of 1.0053 at 393 K (Table 2, corrects to 1.0070 at 298 K).

In summary, the authors are able to correlate a normal IE with "no bond order changes" at N1, while an inverse IE indicates formation of an intermediate prior to decarboxylation that incurs a bond order change at N1. The decarboxylation of OMP in ODCase is measured by these authors to be 1.0068 at 298 K (Table 2), which indicates no bond order change at N1 throughout the enzyme-catalyzed reaction. Such an IE is consistent with direct decarboxylation, where no proton transfer is involved, or with a mechanism involving decarboxylation with protonation at O4. The authors argue that the normal IE appears *inconsistent* with O2 protonation, in that they expect protonation at O2 to result in a bond order change at N1. We sought to lend more insight into the interpretation of these experimental IE's by conducting IE calculations for various potential mechanisms for comparison to Cleland's values.

Toward that end, we have calculated the ^{15}N isotope effect at N-1 for the decarboxylations of picolinic acid (**9**), *N*-methyl picolinic acid (**12**), 1-methyl orotate (**1b**), 2-protonated-1-methyl orotate (**3b**), and 4-protonated-1-methyl orotate (**7b**). As a control, we also calculated the IE for the protonation of pyridine.[46] Our results are summarized in Table 2.

(45) The authors measured an isotope effect of 1.0036, and derive an "intrinsic" isotope effect of 1.0068, by accounting for "the tendency of the substrate to react forward once bound to the enzyme rather than dissociate".

(46) Kurz, J. L.; Daniels, M. W.; Cook, K. S.; Nasr, M. M. *J. Phys. Chem.* **1986**, *90*, 5357−5360.

Table 2. ^{15}N−N1 Decarboxylation Isotope Effects[a]

reaction	exptl[b]	calcd
picolinic acid **9** → **11** + CO_2	0.9955 ± 0.0004[c]	0.9907[c]
N-methyl picolinic acid **12** → **14** + CO_2	1.0053 ± 0.0002[d]	1.0047[d]
OMP in ODCase OMP → UMP + CO_2	1.0068 ± 0.0007	
1-methyl orotate **1b** → **2b** + CO_2		1.0069
1-methyl orotate, 2-protonated path (ylide mech.) **1b** → **4b** + CO_2		1.0043
1-methyl orotate, 4-protonated path (carbene mech.) **1b** → **6b** + CO_2		1.0054
pyridine protonation	0.9793 ± 0.0007	0.9721

[a] Temperature is 298 K unless otherwise noted. [b] Reference 36. [c] Measured/calculated at 463 K. [d] Measured/calculated at 393 K.

The calculations of picolinic acid and *N*-methyl picolinic acid yield ^{15}N1 isotope effects of 0.9907 (463K) and 1.0047 (393 K), respectively. These compare favorably to the experimental values of 0.9955 and 1.0053. Furthermore, because the picolinic acid reactions include the key proton-transfer equilibrium, we also calculated the pyridine protonation IE, which we found to be 0.9721 at 298 K, compared to 0.9793 experimentally.[46−48]

Having established the validity of our calculations, we explored the decarboxylations of the 1-methyl orotate species. The decarboxylation of 1-methyl orotate itself (**1b**) has a calculated IE value of 1.0069 (Table 2). Such a decarboxylation could correspond to the uncatalyzed decarboxylation, or a catalyzed, direct decarboxylation mechanism in which the N1 remains ternary throughout the transformation. The calculated IE value of 1.0069 is consistent with the ODCase-catalyzed value of 1.0068.

However, of real interest to us is how the protonation mechanisms would be affected by isotopic substitution. A priori, it did not seem obvious to us whether protonation at O2 or at O4 would cause a "bond order change" at N1. The examples of picolinic acid and pyridine are fairly straightforward, in that the nitrogen itself is protonated, but what does a "bond order change" mean when protonation occurs at a distant, but related, site such as one of the oxygens?

We find that 2-protonation/decarboxylation (**1b** → **4b** + CO_2) results in a calculated decarboxylation IE of 1.0043 (Table 2). The 4-protonation/decarboxylation mechanism (**1b** → **6b** + CO_2) has a calculated IE of 1.0054 (Table 2). Therefore, protonation at neither O2 nor O4 has a significant enough effect on the "bond order" of the N1 to cancel out the normal decarboxylation IE. The equilibrium IE for protonation on the 2-oxygen of 1-methyl orotate is calculated to be 0.9972, much less significant than that for the protonation of pyridine (0.9721) or for the equilibrium of picolinic acid (**9**) and its N-protonated zwitterion isomer **10** (0.9741). The decarboxylation IE of 1.0071 ultimately swamps out this minor inverse IE. As predicted by Cleland et al., the ^{15}N−N1 IE for protonating the 4-oxygen of 1-methyl orotate is essentially unity; we calculate 1.0004. The subsequent decarboxylation step has an IE of 1.0048, resulting in a overall IE for the reaction of 1.0054.

The small bond order changes at N1 incurred by protonation of the oxygens can be seen in the optimized structures of the 1-methyl orotates (Figure 3). Bond lengths are shown in angstroms. When 1-methyl orotate is protonated on the 2-oxygen, although the N1−C2 bond shortens somewhat (from 1.392 to 1.330 Å, a delta (Δ) of −0.062 Å), the N1−C6 bond lengthens (Δ = +0.028 Å). The N1−Me bond changes slightly as well, from 1.479 to 1.497 Å (Δ = +0.018 Å). The "overall change" in bonding around N1 can be roughly calculated by adding the deltas; $\sum\Delta$ = −0.016 Å. In the case of 4-protonation, the N1−C2 and N1−Me bonds lengthen slightly, while the N1−C6 bond shortens; the sum of the bond deltas, $\sum\Delta$, is −0.004 Å. As a point of comparison, we have also included the equilibrium of picolinic acid (**9**) with its N-protonated zwitterion picolinate isomer (**10**). The N−C6 bond changes from 1.345 to 1.352 Å (Δ = +0.007), while the N−C2 bond stays 1.353 Å in both structures. The N−H bond does not exist in picolinic acid, and has a bond length of 1.039 Å in the zwitterion (Δ = +1.039 Å). The sum of the bond deltas in this case is $\sum\Delta$ = +1.046 Å. Thus, quantum mechanical calculations indicate that there is surprisingly little change in the overall bond order at N1 upon protonation of the oxygens; the overall change is miniscule compared to the bond order change incurred by direct protonation of the nitrogen in picolinic acid, and this is reflected in the small inverse isotope effects associated with oxygen protonation.

Calculations therefore indicate that direct decarboxylation, the ylide mechanism (O2 protonation), and the carbene mechanism (O4 protonation) are all consistent with the observed experimental isotope effect. In summary, ^{15}N isotope effects at N1 cannot distinguish a direct decarboxylation mechanism from one involving protonation to either oxygen.

With regard to the "ground state destabilization" hypothesis, if a direct decarboxylation mechanism is in effect, then ground state destabilization could be responsible for catalysis. We, however, are interested in the physical option of a direct decarboxylation; destabilization is merely an explanation of how catalysis could occur if decarboxylation did not involve proton transfer or some other means of acceleration.[26,49,50]

These results beg the question: Can any of the possible ODCase mechanisms be differentiated by isotope effects? There is substantial precedence for the accuracy of isotope effect calculations, and the computational ability to calculate quantitatively what isotope effects should be.[17,51−56] We predict that ^{15}N IE's at N3 could potentially differentiate among the ylide

(47) Haake, P.; Mantecon, J. *J. Am. Chem. Soc.* **1964**, *86*, 5230−5234.

(48) Stephenson, H. P.; Sponer, H. *J. Am. Chem. Soc.* **1957**, *79*, 2050−2056.

(49) One issue with protonation on the O2 or the O4 that should be mentioned is the availability of a proton donor. The crystal structures do not indicate close proximity of the key lysine to either oxygen, and the oxygens are also hydrogen bonded to amide groups. However, the lysine could still move near the O4 to effect proton transfer in the dynamic protein structure, or protonation may occur through a lysine−water bridge.

(50) Lysine−water bridge proposal attributable to Dean J. Tantillo and Bruce N. Hietbrink.

(51) Singleton, D. A.; Thomas, A. A. *J. Am. Chem. Soc.* **1995**, *117*, 9357−9358.

(52) Singleton, D. A.; Merrigan, S. R.; Beno, B. R.; Houk, K. N. *Tetrahedron Lett.* **1999**, *40*, 5817−5821.

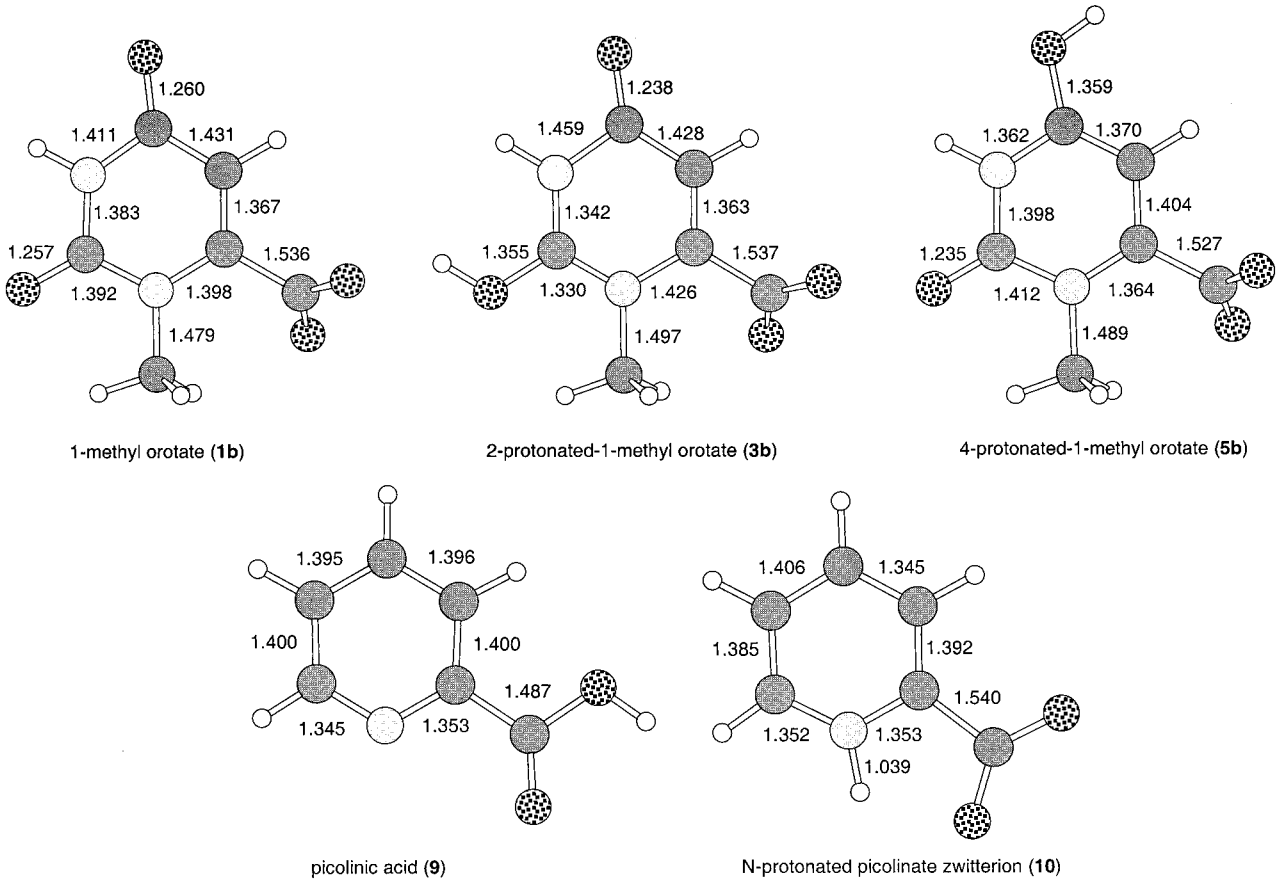
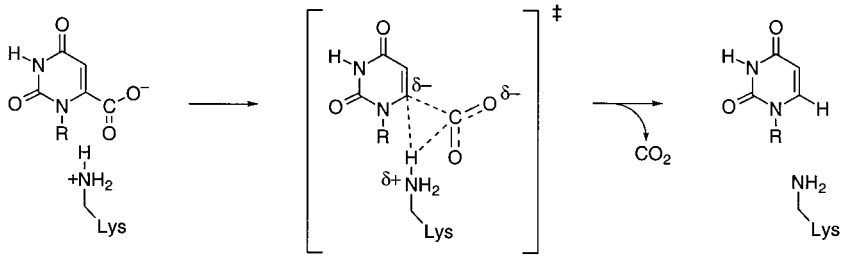


Figure 3. B3LYP/6-311++G optimized structures of 1-methyl orotate, 2-protonated-1-methyl orotate, and 4-protonated-1-methyl orotate. Calculated bond lengths, in Å, are indicated.

Scheme 3



mechanism, the carbene mechanism, and direct decarboxylation. Our computational results for ^{15}N IE's at the N3 position are summarized in Table 3. Our theoretical prediction is that direct decarboxylation without proton transfer (modeled by the first reaction, **1b** → **2b** + CO_2) should yield a ^{15}N3 IE greater than 1 (predicted value: 1.0014). The 2-protonation mechanism (**1b** → **4b** + CO_2) should also yield a ^{15}N3 IE greater than 1 (predicted value: 1.0027). In contrast, the 4-protonation mechanism (**1b** → **6b** + CO_2) should yield a ^{15}N3 IE less than 1 (predicted value: 0.9949). Thus, the 4-protonation mechanism could potentially be differentiated from the direct decarboxylation and 2-protonation mechanisms through N3 isotope effect studies. Experimental studies testing this prediction are currently underway.

Table 3. Calculated ^{15}N−N3 Decarboxylation Isotope Effects[a]

reaction	calculated
1-methyl orotate **1b** → **2b** + CO_2	1.0014
1-methyl orotate, 2-protonated path **1b** → **4b** + CO_2	1.0027
1-methyl orotate, 4-protonated path **1b** → **6b** + CO_2	0.9949

[a] Temperature is 298 K.

Conclusions

The mechanism of OMP catalysis remains in question, and certainly, more data are needed. However, we have shown that energetically, protonation, whether on O2 or on O4, is still a real possibility as a viable OMP catalytic mechanism, and that 4-protonation is favored over 2-protonation, both intrinsically and because of the greater basicity of the 4-oxygen. Furthermore, N1 isotope effects cannot distinguish among several of the likely mechanisms, and therefore protonation cannot be ruled out. The possibility also remains, as proposed by Ealick and Begley et

(53) Keating, A. E.; Merrigan, S. R.; Singleton, D. A.; Houk, K. N. *J. Am. Chem. Soc.* **1999**, *121*, 3933−3938.
(54) DelMonte, A. J.; Haller, J.; Houk, K. N.; Sharpless, K. B.; Singleton, D. A.; Strassner, T.; Thomas, A. A. *J. Am. Chem. Soc.* **1997**, *119*, 9907−9908.
(55) Singleton, D. A.; Merrigan, S. R.; Liu, J.; Houk, K. N. *J. Am. Chem. Soc.* **1997**, *119*, 3385−3386.
(56) Beno, B. R.; Houk, K. N.; Singleton, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 9984−9985.

al., that proton transfer occurs directly onto C6, in an S_E2-type mechanism (Scheme 3).[22,26,30] Intuitively, this mechanism would also be consistent with Cleland's isotope effect values; attempts to find the transition state for this viable pathway were stymied by the computational tendency of the proton to protonate the carboxylate oxygens rather than C6. We also looked for the intermediate of this reaction (**15**), but it is not a stationary point on the potential energy surface, and immediately falls apart to product, which means that energetically, the Ealick−Begley mechanism is very attractive—the reaction is probably very fast. Further studies of this mechanism are planned. Last, we have made a theoretical prediction that ^{15}N isotope effects at N3 will be able to discriminate 4-protonation from 2-protonation and direct decarboxylation mechanisms. Experimental and further computational studies are underway to test this prediction and to uncover the mechanism of ODCase.

Acknowledgment. We thank the National Science Foundation (CHE-0092215), ACS-PRF (Grant No. 32732-G4), the Rutgers Environmental Health Sciences Exploratory Research Grant Program, and the Rutgers Busch Grant Program for support, and the National Center for Supercomputing Applications, the High Performance Computing Center at the University of Kentucky, and the Boston University Scientific Computing Facilities for computational resources. We are also grateful to Professors Peter Beak, Ken Houk, Dan Singleton, and Dr. Dean Tantillo for valuable discussions.

Supporting Information Available: Energies (in hartrees) and Cartesian coordinates for the optimized structures of **1**−**6** and **8**−**14** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0117332