Communication

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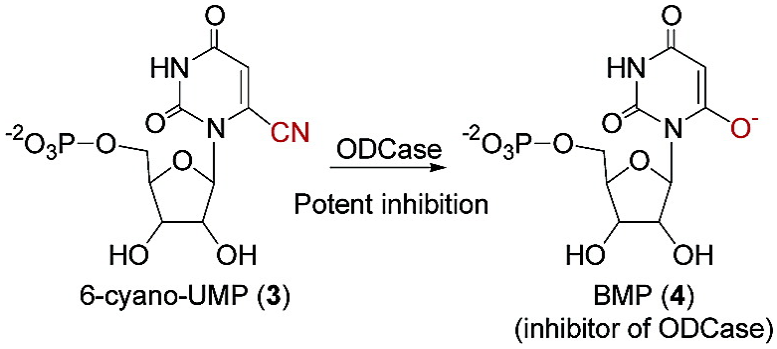

6-cyano-UMP (**3**)

BMP (**4**)
(inhibitor of ODCase)

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An Unprecedented Twist to ODCase Catalytic Activity

Masahiro Fujihashi,[#,‡,@,¶] Angelica M. Bello,[#,+] Ewa Poduch,[#,+] Lianhu Wei,[#,+] Subhash C. Annedi,[#,+] Emil F. Pai,[*,#,‡,§,@] and Lakshmi P. Kotra[*,#,+,†]

Molecular Design and Information Technology Center, Leslie Dan Faculty of Pharmacy, 19 Russell Street, Toronto, ON M5S 2S2 Canada, Department of Chemistry, University of Toronto, Departments of Medical Biophysics, Biochemistry, and Molecular and Medical Genetics, 1 King's College Circle, Toronto, ON M5S 1A8, Canada, and Division of Cancer Genomics and Proteomics, Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, ON M5G 2M9, Canada

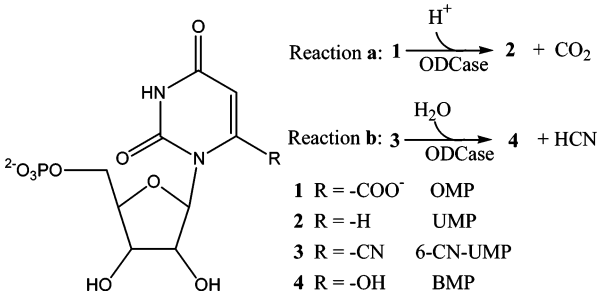
Received July 20, 2005; E-mail: p.kotra@utoronto.ca; pai@hera.med.utoronto.ca

Enzymes have evolved to specifically carry out well-defined chemical reactions, usually enhancing the reaction rate by several orders of magnitude.[1,2] It is, however, not too uncommon in Nature to find enzymes promiscuous in the choice of their substrates.[3] Orotidine-5′-monophosphate decarboxylase (ODCase) is involved in the de novo biosynthesis of uridine-5′-monophosphate (UMP) from orotidine monophosphate (OMP) and is one of the most proficient enzymes currently known (Scheme 1, Reaction **a**).[3−5] The half-life of the substrate in neutral aqueous solution is about 78 million years, but when catalyzed by ODCase, this changes to 18 ms. The enzyme's proficiency ($[k_{cat}/K_M]/k_{non}$) is ca. 10^{23} M^{-1}.[4] The enormous acceleration of this decarboxylation reaction is achieved without the help of any cofactors, metal ions, delocalization effects, or intermittent covalent bond formation, generating wide interest in this enzyme's elusive mechanism.[4]

Extensive investigations using structural biology, kinetics, and computational chemistry support the idea that elimination of CO_2 from OMP is followed by protonation of the electron-rich C6 position to generate the product UMP. Presently, the most discussed mechanisms of generating this electron-rich species involve either a protonation/zwitterion species or electrostatic plus steric stress leading to an anionic species on the pyrimidine ring.[4,6]

Several X-ray structures of ODCases from four different species, native structures, and mutants complexed with 6-aza-uridine-5′-monophosphate (6-aza-UMP), 6-hydroxyuridine-5′-monophosphate (barbiturate-5′-monophosphate, BMP (**4**)), and UMP (**2**) all indicate that the active site is very tightly packed.[6,7] These structures do not seem to be consistent with protonation of the orotidine ring due to the absence, in the ODCase's catalytic pocket, of appropriate residues in the vicinity of O2 and O4 of the pyrimidine ring.[6,7c−e] The bases of other nucleotides, such as cytosine monophosphate (CMP) or xanthosine monophosphate (XMP), are seen to bind outside the active site cavity, although the phosphoribosyl moiety assumes a position almost identical to that of UMP.[8] The two most potent inhibitors of ODCase, BMP (**4**, $K_i = 8.8 \times 10^{-12}$ M) and 6-aza-UMP ($K_i = 5.5 \times 10^{-7}$M), are proposed to mimic the presumed C6 carbanion transition-state species. During our investigations of 6-CN-UMP (**3**) probing the effects of the presence and location of the negative charge around the C6 position, we discovered a yet-uncovered potential of ODCase. The cyano group was also anticipated to mimic an "electron-rich" moiety, reminiscent of the substrate's carboxylate group. However, an in-depth analysis revealed what appeared to contradict the general perception.

Scheme 1



Given the enzyme's reliance on the standard amino acids as well as its extreme proficiency, one might assume that ODCase represents the ultimate in specialization. Here, however, we present evidence that ODCase, which has evolved to carry out a decarboxylation reaction most probably via a *nucleophilic* intermediate, is also able to catalyze a (pseudo) hydrolysis, possibly via induction of an *electrophilic* species.

ODCase (from *Methanobacterium thermoautotrophicum)* incubated with 6-cyano-UMP (**3**), a substrate analogue originally thought to be inert to enzyme action, lost its activity in a time-dependent and unrecoverable manner. When crystals taken from this incubation mixture after 7 days were analyzed by X-ray crystallography, BMP (**4**) was bound in the active site of ODCase (Figure 1). The quality of the corresponding electron density map (resolution = 1.45 Å) was sufficient to identify the location of carbon, nitrogen, and oxygen atoms with high confidence (Figure 1A, and Movie S1 in Supporting Information). When crystals were grown in the presence of 5 mM compound **3**, flash-frozen, and analyzed by X-ray crystallography 36 h after mixing of the solutions, the "substrate" **3** was bound to the active site in a manner analogous to that of UMP (**2**), 6-aza-UMP, and BMP (**4**) (Fujihashi et al., manuscript in preparation).In further experiments, mass spectral analyses of ODCase mixed with **3** at 0 h showed a peak at 27 694.0 amu (peak D, Figure 2A), indicating the formation of a weak complex of ODCase (M.W. = 27 344.6) and **3** (M.W. = 349.4) (Figure 2A). After 7 days of incubation of **3** with ODCase, mass spectral analyses revealed a peak at 27 685.7 amu (peak B, Figure 2B). This peak corresponds to the molecular weight of the complex of ODCase (M.W. = 27 344.8) and **4** (M.W. = 340.9), indicating the transformation of **3** into **4** (Scheme 1, Reaction **b**; also see Supporting Information Figure S1B). The peak height of the complex of ODCase and **4** (peak B, Figure 2B), which is a tight complex ($K_i \sim 10^{-12}$ M), is over 50% of the height of the largest peak. Additionally, incubation of compound **3** in the buffer for 7

[#] Molecular Design and Information Technology Center.
[+] Leslie Dan Faculty of Pharmacy.
[†] Department of Chemistry, University of Toronto.
[‡] Department of Biophysics
[§] Department of Biochemistry, and Molecular and Medical Genetics.
[@] Division of Cancer Genomics and Proteomics.
[¶] Current address: Graduate School of Science, Department of Chemistry, Kyoto University, Sakyo, Kyoto 606-8502, Japan.

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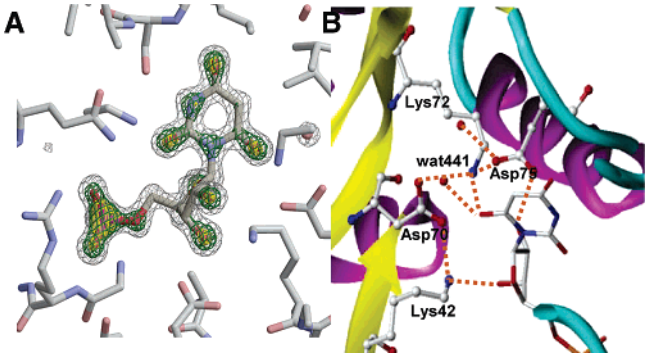


Figure 1. (A) 1.45 Å resolution electron density map of BMP in the active site of ODCase in crystals in contact with solutions of compound **3** for 7 days. The densities are shown at σ levels at 14.6, 11.6, and 5.0 (yellow, green, and gray, respectively). O, N, and C atoms can be clearly distinguished. (B) Hydrogen bonding network between the ligand BMP and the active site of ODCase, depicting the multiple interactions near the C6 position.

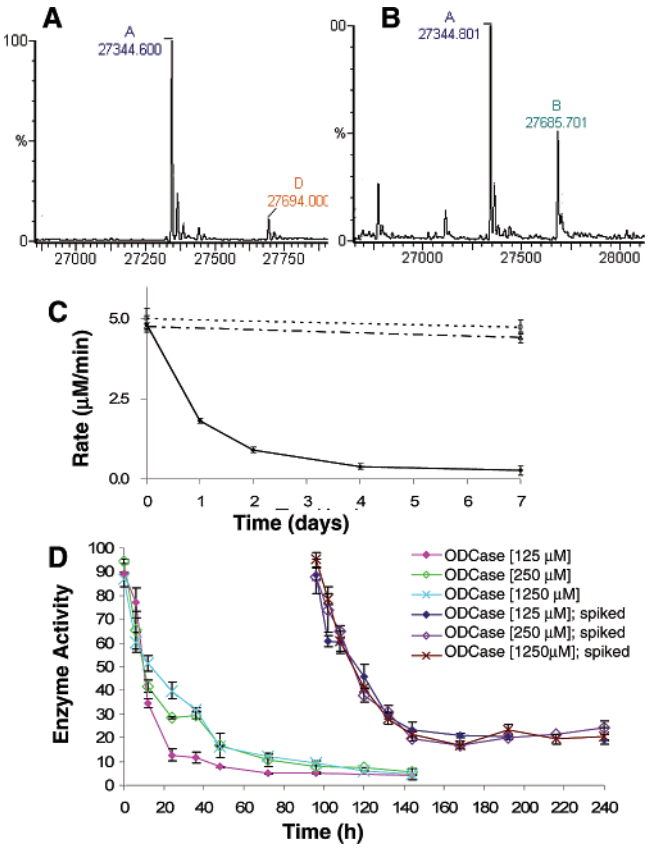


Figure 2. Mass spectral analyses of ODCase with **3** (for 0 h and 7 day incubations at room temperature shown in panels A and B, respectively). A shift in the peaks of ODCase from 27 344.6 to 27 694.0 (peaks A and D, panel A) is due to the formation of the ODCase complex and with **3** (M.W. shows the formation of the complex between ODCase and **4**. Panel C shows the loss of activity (as measured by the rate of conversion of OMP to UMP) of ODCase incubated in the presence of **3** (—) and in the absence of **3** (- - -). In the absence of ODCase, compound **4** is not generated from compound **3** in the incubation mixture (-·-·-). Panel D shows the enzyme activity of ODCase at three concentrations (125, 250, and 1250 μm) when incubated with 100× concentration of compound **3**. At 96 h after incubation, a portion of all three samples was spiked with fresh ODCase, and their activities were monitored separately.

days did not show any traces of compound **4** (Supporting Information, Figure S2), eliminating the possibility of a "chemical conversion" of **3** to **4**. When compound **3** was incubated with ODCase, a time-dependent loss of activity over 2−3 days was observed,

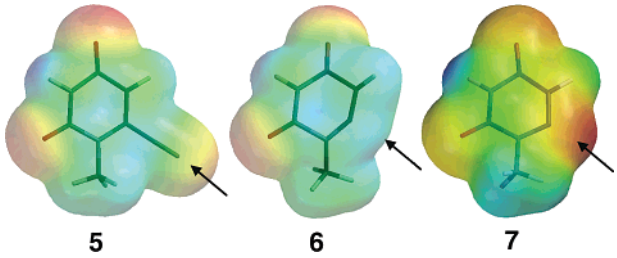


Figure 3. Electrostatic potential for **5**, **6**, and **7** mapped onto their respective electron density surfaces (electronegative potential is shown in red and electropositive in blue). The C6 position is marked by an arrow.

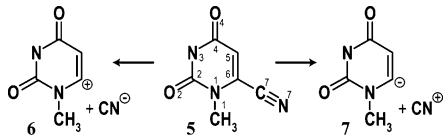
indicating an enzymatic conversion of **3** to **4**, which is a very potent inhibitor of ODCase (Figure 2C) and inactivates ODCase very efficiently. Thus, the enzymatic assay with controls (Figure 2C) affirms the inactivation of ODCase by a product from compound **3**, but not any contaminant including compound **4**, which would have inactivated ODCase immediately.

Enzymatic assays that show increasing time-dependent, irreversible inhibition of ODCase when incubated with **3** (Figure 2C), mass spectral analyses of the complexes of the enzyme's substrate (**3**) and product (**4**) (Figure 2A and B, and Supporting Information, Figure S1B), and the X-ray crystal structures (Figure 1) all together provide compelling evidence for the substitution of the 6-cyano group with a hydroxyl moiety and its catalysis by ODCase at its active site.

To further confirm that the transformation of compound **3** into compound **4** is carried out by ODCase, but not by any other undetectable levels of a contaminant enzyme in the ODCase preparation, we undertook several concentration-dependent spiking experiments. Three different high concentrations of ODCase at 125, 250, and 1250 μM were incubated with 100× the concentration of compound **3** (1.25, 2.5, and 12.5 mM), and the ODCase enzyme activity was monitored. At all three concentrations, the loss of activity was observed almost at the same rate (Figure 2D), and at 72 h, more than 85% of the enzyme activity was lost. This loss of activity is, based on the evidence thus far, due to the conversion of compound **3** into compound **4**, a very efficient inhibitor of ODCase. If there were another contaminant enzyme, at undetectable levels but with a high rate of conversion of compound **3** into compound **4**, one would anticipate a linear rate of loss of activity of ODCase. However, ODCase does not show a linear rate of loss of activity at all three different concentrations (Figure 2D). Additionally, this contaminant enzyme would be continuously producing compound **4** from compound **3** given the large excess of compound **3**. Thus, an additional "spike" of ODCase after 96 h would be expected to lose enzyme activity immediately, should there be compound **4** freely available in the incubation mixture. However, when these experiments were performed, the rate of loss of activity was very similar to that observed at $t = 0$ h (Figure 2D, profiles in blue, violet, and brown). This indicated that there is no additional free compound **4** in the solution of the incubation mixture, confirming the conclusion that ODCase does, in fact, catalyze the transformation of compound **3** into **4**.

Samples of the three incubation mixtures at 144 h (Figure 2D, magenta, green, and cyan profiles) were filtered to remove the enzyme from the incubation mixture using an Ultrafree −0.5 Centrifuge Filter Unit (5 kDa MWCO, Millipore), and the filtrate was analyzed by high-resolution mass spectrometry to detect any traces of compound **4**. However, there was no indication of compound **4** in these samples, and only compound **3** was detected (Supporting Information, Figure S3). These analyses indicated that compound **4** that is generated in the incubation mixture is bound

Scheme 2



to the ODCase enzyme, and there is no free compound **4** in the solution. All of the above evidence supports the unusual and exciting conclusion that the transformation of compound **3** into **4** is indeed catalyzed by ODCase.

Contrary to the natural biochemical transformation of OMP to UMP, which is thought to progress through a *nucleophilic* intermediate, the transformation of **3** to **4** possibly may include an *electrophilic* center. This would further react with a nucleophilic water molecule, producing **4**. The two other alternatives, either $CN^{\bullet}$ radical generation (homolytic cleavage of the C6−CN bond) or the generation of a CN^{+} cation, seem not plausible. We used 1-methyl-6-cyanouracil (**5**) as a model compound and conducted computational investigations. Hypothetical species **6** and **7** were used to evaluate the plausibility of elimination of the cyano moiety from **5** in its anionic versus cationic form (Scheme 2; see Supporting Information, Tables S2 and S3). Optimized geometries for **5** and **7** indicated that the uracil ring is planar in these two species, in contrast to the uracil ring in **6**. Electrostatic potentials for **5**, **6**, and **7** mapped onto their respective electron density surfaces indicate an electropositive character at the C6 position for compound **6** and an electronegative character for **7**. Generation of the cationic species **6** is energetically favored ($\Delta H = 232.0$ kcal/mol) in comparison to the generation of the anionic species **7** ($\Delta H = 413.6$ kcal/mol) from compound **5**. Although performed in the gas phase, the calculations clearly show a preference for the involvement of the cationic species **6**. *If one considers that ODCase is able to accept compound **3** as a substrate* and generate compound **4**—and in fact all of the biochemical, structural, and enzymatic evidence does support this process—although very slowly in comparison to its natural enzymic reaction, the most probable pathway for this new reaction may be through a species such as **6**. Other mechanisms such as a Michael addition at the C6 position of compound **3** by a water molecule generating an sp^3 center at C6 stabilized by a hydrogen bond at O4, followed by the elimination of the cyano moiety, are plausible. However, with our current understanding of the binding site of OMP in ODCase and its interactions with various nucleotide derivatives, it is unlikely that such a pathway is adopted in the biotransformation of compound **3** to **4** by ODCase. We did not observe any formation of covalent intermediates, either in the high-resolution mass spectral analyses or in the several X-ray structures of the ODCase in the presence of compound **3**, practically eliminating the possibility of covalent catalysis for the formation of compound **4**.

Here, we describe a hitherto unknown reaction for a highly proficient member of the enzyme world, ODCase, a reaction which incidentally also leads to its own inhibition. If we accept that, in the case of ODCase, electrostatic and/or steric stress applied to its biological substrate contributes to decarboxylation,[6] then a similar "stress" applied to a bioisosteric group (cyano instead of carboxyl, both having a similar length) of a compound not encountered in Nature can lead to an inhibitory product. This "new" potential of ODCase of establishing a potential (partial) electrophilic center at the C6 position of the aromatic base creates opportunities for the design of novel mechanism-based inhibitors.

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Supporting Information Available: Atomic coordinates of the BMP-ODCase complex have been deposited into the Protein Data Bank under code 1X1Z. Details on the synthesis of compound **3**; mass spectra of ODCase alone, ODCase incubated with the mixture of compounds **3** and **4**, and compound **3** after incubation for 7 days in the buffer; atomic charges, ESP surfaces and optimized geometries for **5**, **6**, and **7**; details of crystallization, structure determination, and enzyme kinetics; movie of the electron density map. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pauling, L. *Nature* **1948**, *167*, 707−709.
(2) Silverman, R. B. *The Organic Chemistry of Enzyme Catalyzed Reactions*; Academic Press: San Diego, CA, 2002.
(3) O'Brien, P. J.; Herschlag, D. *Chem. Biol.* **1999**, *6*, R91−R105.
(4) Warshel, A.; Florian, J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5950−5955.
(5) (a) Snider, M. J.; Wolfenden, R. *J. Am. Chem. Soc.* **2000**, *122*, 11507−11508. (b) Radzika, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93. (c) Miller, B. G.; Wolfenden, R. *Annu. Rev. Biochem.* **2002**, *71*, 847−885. (d) Sievers, A.; Wolfenden, R. *J. Am. Chem. Soc.* **2002**, *124*, 13986−13987.
(6) Wu, N.; Mo, Y.; Gao, J.; Pai, E. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2017−2022.
(7) (a) Harris, P.; Poulsen, J. C. N.; Jensen, K. F.; Larsen, S. *J. Mol. Biol.* **2002**, *318*, 1019−1029. (b) Miller, B. G.; Butterfoss, G. L.; Short, S. A.; Wolfenden, R. *Biochemistry* **2001**, *40*, 6227−6232. (c) Harris, P.; Poulsen, J. C. N.; Jensen, K. F.; Larsen, S. *Biochemistry* **2000**, *39*, 4217−4224. (d) Appleby, T. C.; Kinsland, C.; Begley, T. P.; Ealick, S. E. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2005−2010. (e) 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.
(8) (a) Wu, N.; Pai, E. F. *Biochemistry* **2002**, *41*, 4002. (b) Wu, N.; Gillon, W.; Pai, E. F. *J. Biol. Chem.* **2002**, *277*, 28080.

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