Crystal Structures of Orotidine Monophosphate Decarboxylase: Does the Structure Reveal the Mechanism of Nature's Most Proficient Enzyme?

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Introduction

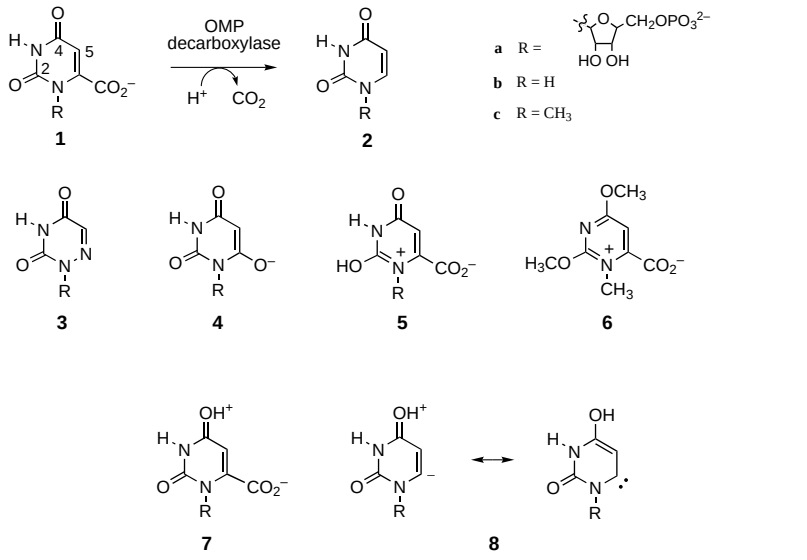
The decarboxylation of orotidine 5′-monophosphate (OMP, **1a**) to form uridine 5′-monophosphate (UMP, **2a**) by orotidine 5′-monophosphate decarboxylase (ODCase) is an essential step in nucleic acid biosynthesis (Scheme 1).[1] The conversion of **1** to **2** is very unusual, in that all other biochemical decarboxylations involve resonance stabilization of a carbanion formed by loss of CO_2 from a carboxylate.[1, 2] ODCase achieved star status when Radzicka and Wolfenden reported that it is the most proficient enzyme known;[3] $(k_{cat}/K_m)/k_{uncat}$ is an astonishing $2.0 \times 10^{23}\,M^{-1}$!

Because of its mechanistic uniqueness and the world's record for acceleration of a reaction, the mechanism of ODCase has attracted much interest. Until last year, no structural data were available about this enzyme, but recently four different X-ray crystallographic structures were reported nearly simultaneously. The authors of these studies made various new proposals about mechanisms, some quite unusual and all rather tentative. This article describes this work and suggests further mechanistic possibilities.

Mechanistic proposals

Many studies of the enzyme and model systems have been explored to try to understand how this decarboxylation occurs. Occasionally conflicting experimental results and some debate eventually led to the conclusion that cofactors and metal ions do not play a role in catalysis.[4, 5] Transition state analogues, such as 6-azauridylate (6-azaUMP, **3a**) and barbituric acid ribonucleotide (BMP, **4a**), are particularly effective inhibitors of yeast ODCase ($K_i = 5.1 \times 10^{-7}$ and $8.8 \times 10^{-12}\,M$, respectively).[6, 7] Before the year 2000, no crystal structure existed in spite of valiant attempts.[8] Studies of the enzyme mechanism by Jones and Smiley indicate that Lys93 (in the yeast enzyme) is important for catalysis, but not for binding.[9] The V_{max}/K_m value of ODCase is pH-dependent, with a maximum at pH 7; this result has been interpreted to indicate that the enzyme possesses a catalytic group which has a pK_a value of ca. 7.[9, 10] Catalytic antibodies that catalyze the decarboxylation of orotate have also been developed.[11, 12]

Various mechanistic hypotheses have been proposed to explain the enormous catalysis by ODCase. Silverman and coworkers suggested a covalent mechanism



Scheme 1. *The reaction catalyzed by ODCase (**1a** → **2a**), model systems (**1b**, **1c**), inhibitors of the enzyme (**3**, **4**), the Beak intermediate **5**[6] and a model compound (**6**), and the O4-protonated orotate (**7**) and the product formed upon its decarboxylation (**8**).*

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involving nucleophilic attack at C5,[13] but this was subsequently shown by ^{13}C and D isotope effects to be unlikely.[10, 14] Beak and co-workers examined the decarboxylation of 1,3-dimethylorotic acid in sulfolane and proposed that decarboxylation occurred via a zwitterion analogous to **5**.[6] The fast (4×10^8-fold acceleration over the parent reaction) decarboxylation of the betaine **6** led the authors to propose that the enzyme might effect catalysis by favoring the formation of zwitterion **5**. This mechanism was widely accepted for a long time.[7, 10, 11, 14, 15]

In 1997, two of us (Lee and Houk) conducted calculations that supported the idea of decarboxylation via a zwitterion-like species; however, we showed that the energetically favored pathway in the gas phase involves protonation on the 4-oxygen atom (rather than the 2-oxygen atom) to form zwitterion **7b**.[16] The intermediate formed upon decarboxylation of **7b** (→**8b**) is stabilized as reflected in the carbene resonance structure. Considerations of reactant and product pK_a values[5] (orotate and uracil have pK_a values of ca. 0.5),[16, 17] and the likely nonpolar environment of the ODCase active site led us to suggest that the decarboxylation involves a concerted proton transfer from lysine accompanying decarboxylation to give the stabilized carbene intermediate **8** directly. This mechanism is summarized in Scheme 2.

Since 1997, additional studies have supported or elaborated this mechanism. Wu and co-workers provided kinetic data consistent with the Lee–Houk process.[18, 19] Blanchard and co-workers conducted multiple kinetic isotope effect studies that are consistent with enzymatic decarboxylation through the O4 protonation mechanism, but indicate a stepwise path involving protonation followed by decarboxylation that is quite surprising in light of the pK_a values of Lys93 (ca. 7) versus orotate (ca. 0.5).[17] By using ^{13}C-kinetic isotope effects and theoretical predictions, Singleton, Beak, Lee and co-workers showed that the uncatalyzed, thermal decarboxylation of 1,3-dimethylorotic acid proceeds through O4 protonation.[20]

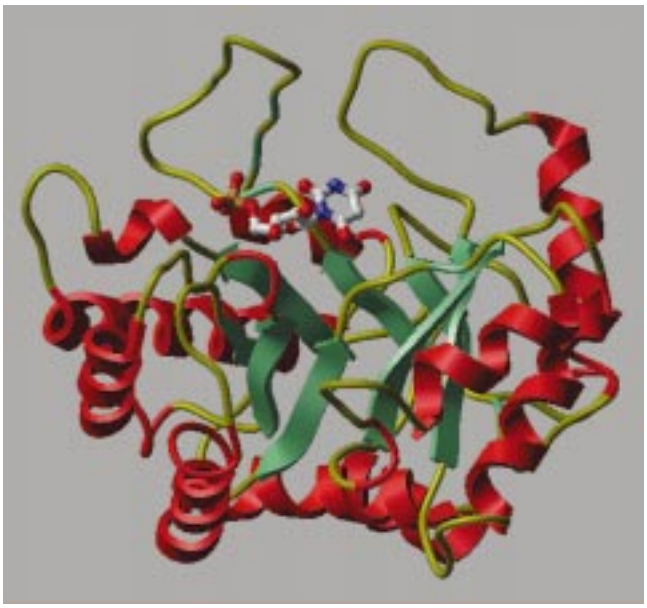
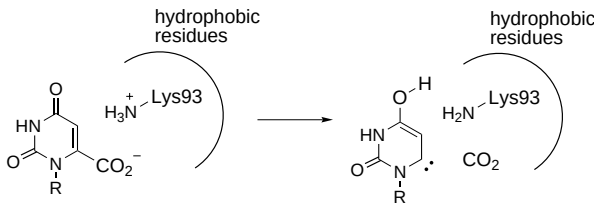


Figure 1. *The inhibitor BMP (**4a**) bound in the active site of ODCase.[22] The enzyme is composed of β-strands (green) and α-helices (red).*



Scheme 2. *The O4 protonation mechanism.*

The crystal structures and new mechanistic proposals

General considerations

Recently, crystal structures of free and inhibitor-bound ODCase from four different species were reported by the groups of Ealick,[21] Short and Wolfenden,[22] Larsen,[23] and Pai and Gao.[24] These structures reveal that ODCase is a dimer consisting of two identical subunits, each of which has a triose-phosphate isomerase (TIM) fold consisting of eight β-strands and eleven α-helices (Figure 1). The active site is located at the end of the TIM barrel and contains residues from both subunits. Based on the structures of free and BMP-bound enzymes, it appears that ODCase may be capable of exhibiting considerable conformational flexibility; due to the combined movements of several loops, the binding site is sealed off from bulk solvent upon complex formation, and this conformational change permits several important binding interactions.[22, 24]

Comparison of the binding sites in each structure reveals striking similarities across species. Although recognition of the sugar and phosphate groups present in each inhibitor as well as in the substrate involves an extensive array of contacts, we will focus on the portion of the active site surrounding the pyrimidine ring, since this is the local environment for the decarboxylation reaction. Figure 2A shows the binding site as found in the crystal structure of the ODCase–BMP complex (from the work of Larsen and co-workers)[23] and the superposition of the binding sites from all four inhibitor-bound structures (Figures 2A and B). Clearly, many identical or similar residues are present in all four complexes, and these share not only locations in sequence, but also in space (Figure 2B). As exemplified by the binding site shown in Figure 2A, inhibitor recognition involves several distinct regions (Figure 2C). The O2/N3/O4 side of the pyrimidine ring makes three hydrogen bonds to uncharged polar residues. The C6 position, which is substituted by a nitrogen atom in 6-azaUMP (**3a**) and bears an attached oxygen atom in BMP (**4a**), is in close

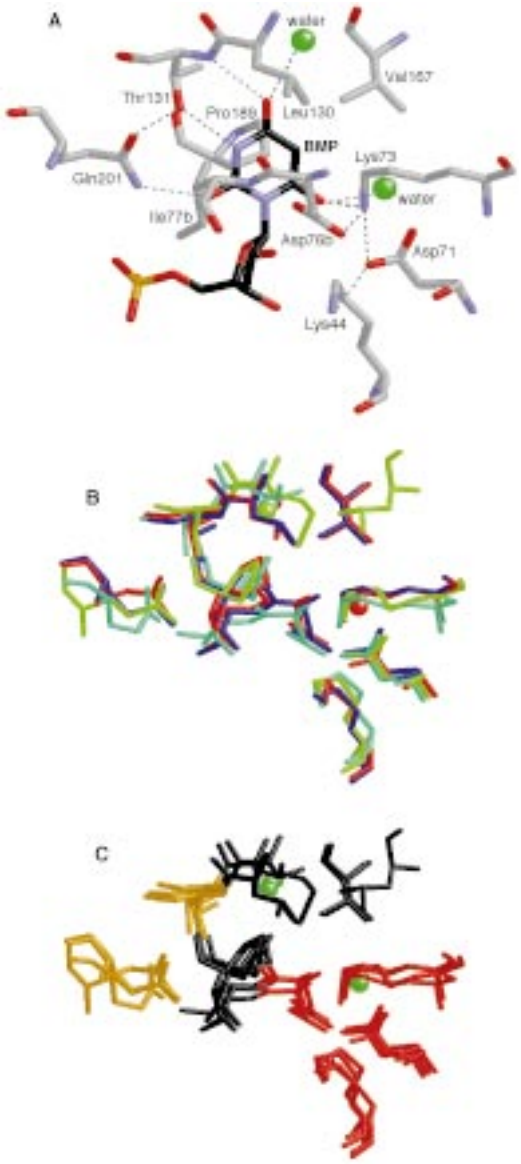


Figure 2. *A: Active-site residues and water molecules in the vicinity of the pyrimidine ring of BMP in the crystal structure determined by Larsen and co-workers.[23] Oxygen atoms are drawn in red, nitrogen atoms in blue, the phosphorous atom in orange, water molecules in green, and carbon atoms in gray for the protein and black for BMP. B: Superposition of the residues shown in Figure 2A (red) with the corresponding residues in the ODCase – inhibitor complexes of Short and Wolfenden et al. (turquoise), Ealick et al. (dark blue), and Pai and Gao et al. (yellow-green). C: The same superposition as in Figure 2B but color-coded by residue type: Charged residues are drawn in red, polar uncharged residues in orange, hydrophobic residues in black, and water molecules in green.*

proximity to a cluster of two aspartates and two lysines. In fact, based on a comparison of ODCase amino acid sequences from more than 80 species, Traut and Temple have noted that this Asp-Lys-Asp-Lys tetrad appears to be absolutely conserved.[25] The π faces of the pyrimidine ring interact with several hydrophobic residues that partially define a pocket that extends beyond and encloses the area around C5. In most of the structures, water molecules are also observed in the vicinity of O2, O4, or both. Selected key interactions for each ODCase – inhibitor complex are shown schematically in Figure 3.

The ODCase – UMP complex

The crystal structure of ODCase from *Bacillus subtilis* with bound UMP—the ultimate product of OMP decarboxylation—has been determined at 2.4 Å resolution by Ealick and co-workers.[21] In this structure, the amide side chain of Gln 194 donates a hydrogen bond to O2 of UMP through its NH_2 group and accepts a hydrogen bond from Thr 123 through the oxygen atom of the amide carbonyl group (Figure 3). The side chain hydroxy group of Thr 123 in turn accepts a hydrogen bond from the proton on N3 of UMP, while O4 on the pyrimidine ring accepts a hydrogen bond from the backbone amide NH of Thr 123. A pocket near C5 of the pyrimidine base consists of several hydrophobic residues (see Figure 2). These interactions are present in all four ODCase – inhibitor structures, with only minor variations.

Although the charged Asp-Lys-Asp-Lys tetrad is in the vicinity of C6, it does not make any direct contacts with bound UMP. Ealick and co-workers suggested that the actual substrate OMP could be destabilized by electrostatic repulsion between its carboxylate group and the side chain carboxylate of Asp 60. This ground state destabilization would be reduced in the transition state as negative charge is shifted from the carboxylate to C6 of the pyrimidine ring, which is close to the protonated ammonium group of the side chain of Lys 62. The authors proposed, therefore, that protonation of C6 occurs in concert with cleavage of the C–C bond in an S_E2 fashion (Scheme 3).

The ODCase – BMP complexes

The crystal structure of the *Saccharomyces cerevisiae* enzyme has been determined in the presence of the inhibitor BMP (**4a**) to 2.4 Å resolution by Short, Wolfenden, and co-workers.[22] The hydroxyenone moiety of BMP is likely ionized in water and in the binding site, since the pK_a values of barbiturates are less than 4. The interactions between the ODCase active site and the C2 – C5 fragment of the barbituric acid ring are analogous to those described above for the UMP complex (Figure 3). However, BMP possesses a recognition element not present in UMP: a (partially) negatively charged oxygen atom attached to C6. Not surprisingly, this charge promotes an additional noncovalent interaction, a hydrogen bond (or salt bridge) between the oxygen and the ammonium group of the side chain of Lys 93. Interestingly, the Asp-Lys-Asp-Lys cluster that provides this interaction assumes a very similar geometry in the UMP-bound, BMP-bound, and inhibitor-free structures, suggesting that this region of the ODCase binding site is somewhat preorganized for the recognition of anionic groups in the vicinity of C6. In addition, water molecules are located in the neighborhood of both O4 and O6.

Based on their analysis of the crystal structure, Short, Wolfenden, and co-workers propose that Lys 93 is oriented so as to stabilize the buildup of negative charge on C6 in the transition state for decarboxylation and to provide the proton that appears at C6 of the product, while not interacting in a favorable way with the substrate carboxylate group.[22] This feature, accompanied by hydrogen bonds from the active site to O2 and O4 which may help delocalize negative charge in the transition state, as well as enhanced interactions with the phosphate portion of OMP during the decarboxylation,[26–28] were invoked as likely sources of catalysis.

A second structure of BMP-bound ODCase—in this case from *Escherichia coli*—was determined at a resolution of 2.5 Å by Larsen and co-workers.[23] The interactions between the active site and

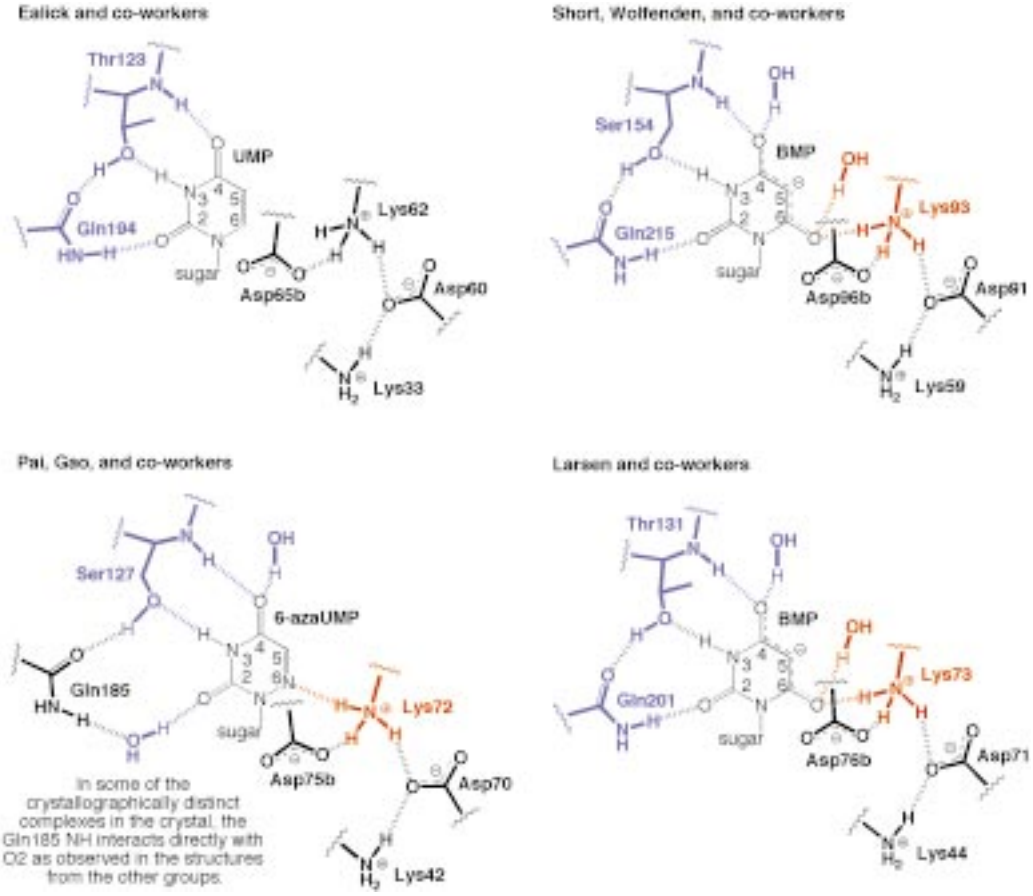
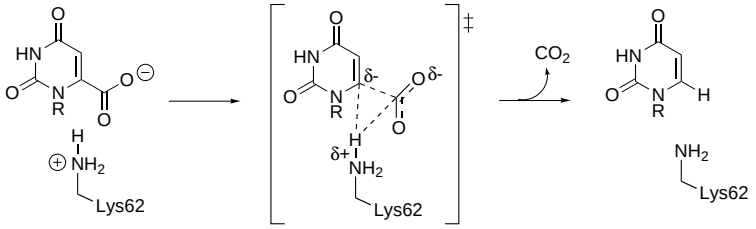


Figure 3. *Key polar interactions between the ODCase active site and bound UMP (**2a**), BMP (**4a**), and 6-azaUMP (**3a**) pyrimidine (or derived) rings, as observed in the crystal structures (determined by the groups of which the principal investigators are listed). Residues that make hydrogen bonding interactions to donors and acceptors in the C2 – C4 region are highlighted in blue, and those that make hydrogen bonding interactions to acceptors in the C6 (N6) region are highlighted in red. All residues are from the same monomeric subunit except Asp residues bearing a "b" label.*



Scheme 3. *The electrophilic substitution (C6 protonation) mechanism.*

the inhibitor in this structure are extremely similar to those observed in the complex of BMP with yeast ODCase (Figures 2 and 3), including the presence of water molecules in the vicinity of both O4 and O6 of bound BMP.

Based on the absence of protein side chains with particularly acidic protons in the vicinity of O2 and O4, and despite the presence of a water molecule near O4, these researchers ruled out mechanisms that call for either O2 or O4 protonation. Instead they proposed two other mechanisms based on the proximity of O6 and Asp 71 (Figures 2 and 3). Their first proposal involved charge repulsion between this aspartate and the carboxylate group of OMP in the enzyme – substrate complex, an unfavorable interaction that might initiate decarboxylation. Their second mechanism involved a short strong hydrogen bond between the carboxy groups of OMP and Asp 71, a favorable interaction that would have to be overcome during decarboxylation. They note, however, that conclusive evidence favoring one or the other mechanism awaits further study.

The ODCase – 6-azaUMP complex

The structure of ODCase from yet another organism—*Methanobacterium thermoautotrophicum*—has been determined at a resolution of 1.5 Å in the presence of another inhibitor, 6-azaUMP (**3a**), by Pai and co-workers[24] (Figure 3). In this case, the hydrogen bonding interaction observed between the key active-site lysine and the oxygen at C6 of BMP is replaced by an analogous interaction between the lysine residue and the lone pair on N6 of 6-azaUMP. The hydrogen bond between O2 and the active-site glutamine amide proton, observed in all of the other ODCase crystal structures, is preserved in some of the complexes found in the

crystal, but an alternative binding mode, in which this interaction is mediated by a bridging water molecule, is observed in others (Figure 3). Other key polar interactions are analogous to those observed in the other structures.

In conjunction with the crystallography performed by the group of Pai, combined quantum mechanics/molecular mechanics (QM/MM) calculations were conducted by the group of Gao.[24] Based on these calculations, which produced activation parameters that agree nicely with those measured experimentally for both the uncatalyzed (i.e. occurring in water) and the enzyme-catalyzed decarboxylation reactions,[26] a mechanism involving considerable destabilization of the OMP ground state was proposed (and referred to as "electrostatic stress", a name for such an effect proposed by Fersht[29] or the "Circe effect" as previously suggested by Jencks[30]). This mechanism is very similar to the one proposed by Larsen and co-workers (see above). In this mechanism, the substrate is destabilized upon binding by bringing its carboxylate group into close proximity with one of the active-site carboxylates (Asp 70, see Figure 3). This raises its energy closer to that of the transition state, thereby reducing the free energy of activation relative to that for the solution reaction (i.e. increasing k_{cat}/k_{uncat}). K_m remains reasonably small (i.e. OMP binds), despite destabilization of the orotate portion of the substrate, because strong stabilizing interactions are present between the enzyme and the sugar phosphate portion of the substrate.

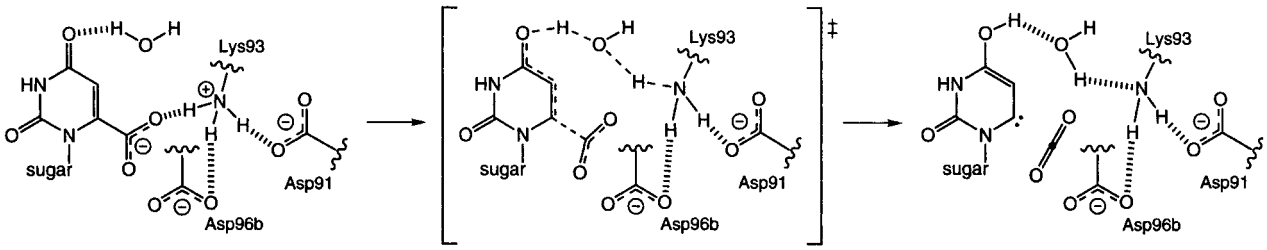
There was a surprising (to us) rush to embrace this mechanism. Wolfenden and co-workers established the great significance of phosphoribosyl binding on k_{cat}/K_m values for this enzyme and cited the "electronic repulsive effects" on the 6-carboxylate.[27, 28] Wu and Gronert et al. voiced support for electrostatic repulsion arguments.[31] Cleland and Rishavy measured ^{15}N isotope effects that indicated that N1 does not undergo a bond order change.[32] This was taken as evidence against O2 protonation but could be consistent with the Lee – Houk mechanism or the electrostatic mechanism. A *Science* editor seized on Cleland's work as the final pronouncement on the mechanism, declaring electrostatic stress the winner![33]

Chemical and Engineering News ran a feature article on the "buzz" associated with ODCase, citing a number of enthusiastic supporters for the electrostatic/Circe mechanism, and only a few, notably Arieh Warshel, as dissenters.[34] In a number of decades of detailed quantitative computational studies, Warshel has established the importance of electrostatic complementarity of the enzyme for the transition state.[35] He has argued forcefully for the importance of oriented dipoles that stabilize the transition state. Indeed, Florián and Warshel previously argued against the Lee – Houk mechanism based upon the premise that the binding site must be highly polar.[36, 40]

Given the possibilities available under biological conditions for stabilizing proximate carboxylates, we believe that any mechanism involving enforced carboxylate – carboxylate repulsion is questionable. Additional calculations on models of the enzyme – substrate complex[37] indicate that either the substrate or active-site carboxylate will be protonated unless they are bridged by the active-site lysine; both of the crystal structures of ODCase – BMP complexes described above do in fact have a lysine bridging between enzyme aspartate carboxylates and the anionic oxygen atom of BMP, and a similar bridging interaction is even seen with the neutral N6 atom of 6-azaUMP (**3 a**). Moreover, ODCase is most effectively inhibited by anionic inhibitors,[38] and a comparison of K_m for decarboxylation and K_i for UMP binding suggests that the anionic substrate is more tightly bound than the neutral product of its decarboxylation by more than an order of magnitude (something desirable for preventing product inhibition).[38, 39] These observations imply that ODCase is actually well suited to make *stabilizing* interactions with anionic substrates.

A variation on the stabilized-carbene theme

If ground state destabilization is not responsible for catalysis, then what is? While catalysis likely involves contributions from the residues contacting both the sugar phosphate group and the N1 – N3 region of OMP, we believe that most of the reduction in activation barrier could result from selective stabilization of the transition state by interactions in the C4 – C6 region. Several water molecules are observed in the ODCase – inhibitor crystal structures (Figures 2 and 3), and we propose that catalysis may actually involve an active-site water molecule. We suggest that before decarboxylation begins, the key lysine residue (Lys 62, Lys 93, Lys 73, or Lys 72, depending on the organism; see Figure 3) bridges the orotate carboxylate and the two active-site aspartates (Scheme 4) in analogy to the ODCase – inhibitor complexes (Figure 3). As the reaction proceeds, a carboxylate – lysine hydrogen bond is broken as a hydrogen bond is formed to an active-site water molecule that bridges to O4. Then, in concert with the transfer of a proton from the lysine to the water molecule, a proton is transferred from the water molecule to O4 of the substrate. Proto-



Scheme 4. *An alternative mechanistic pathway. Residues are numbered as in the yeast ODCase.*

nation of O4 facilitates a redistribution of negative charge away from the carboxylate as the carbon–carbon bond is broken, ultimately resulting in the resonance-stabilized carbene proposed by Lee and Houk.[16] After carbon dioxide loss from the active site, the proton on O4 can be returned to the lysine or shifted to C6, both perhaps via the water molecule.

Summary and conclusions

Despite the similarities among the observed structures, a number of different mechanisms have been proposed to account for ODCase's world-record catalysis. It is clear that additional experimental and theoretical studies will be necessary to pin down the mechanism. The structures are now known, but the mystery of proficiency remains.

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[40] Note added in proof: In a recent publication (A. Warshel, M. Štrajbl, J. Villà, J. Florián, *Biochemistry* **2000**, *39*, 14728) Warshel and co-workers propose a mechanism involving electrostatic stabilization of the transition state.