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Theoretical Studies of the Effect of Thio Substitution on Orotidine Monophosphate Decarboxylase Substrates

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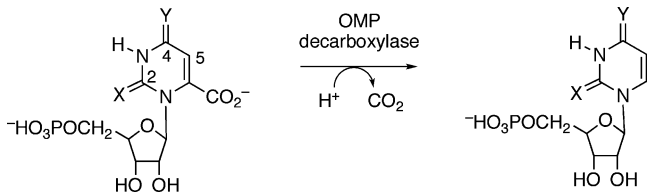

where X=O, Y=O; X=S, Y=O; X=O, Y=S

The effect of replacing carbonyl oxygens with sulfur in a series of orotidine 5′-monophosphate decarboxylase (ODCase) substrates was studied computationally. Previous experimental results indicate that while 2-thio-orotidine 5′-monophosphate (2-thio-OMP) is a poor substrate for ODCase, 4-thio-orotidine 5′-monophosphate (4-thio-OMP) binds to ODCase, and the resultant k_{cat} is measurable. Energetics calculations on 2-thio-1-methyl-orotate and 4-thio-1-methyl-orotate (as models for the 2- and 4-thio-OMPs) indicate that mechanisms involving proton transfer to the 2- or 4-site, regardless of substrate and regardless of whether the 2- or 4-position is a carbonyl or thiocarbonyl, are energetically favorable, as compared to direct decarboxylation without proton transfer. Proton transfer to the 4-site during decarboxylation is found to be energetically more favorable than 2-protonation. Each thiocarbonyl is also found to be more basic than its carbonyl counterpart. Therefore, if 2- or 4-proton transfer is the operative catalytic pathway, energetics alone would not explain why 2-thio-orotidine 5′-monophosphate is a poor ODCase substrate. Conformational preferences for a series of ODCase substrates were also examined computationally. Specifically, the energies and Boltzmann probabilities of the conformers resulting from rotation about the C1′−N1 bond (O4′−C1′−N1−C2 rotation from 0° to 360°) were calculated. It was found that a calculated preference for the syn versus the anti nucleoside conformation correlates to an experimentally better substrate: the OMP and 4-thio-OMP models show a preference for syn conformations, whereas the 2-thio-OMP (the only substrate of the three OMPs that is experimentally found to bind poorly) model shows a preference for an anti conformation. The same rough correlation was found for a series of ODCase inhibitors; that is, a preference for the syn conformation correlates to a better inhibitor. This result is of interest and points to the possibility that the ability for a substrate to bind well to ODCase may be related to its tendency to favor the syn conformation.

Introduction

Orotidine 5′-monophosphate decarboxylase (ODCase) catalyzes the decarboxylation of orotidine 5′-monophosphate (OMP) to form uridine 5′-monophosphate (UMP) (Scheme 1).[1−4] Typically, biological decarboxylations result in resonance stabilization of the product carbanion.[5,6] The decarboxylation of OMP is unusual since the anionic product cannot delocalize into the π system. Interest in this enzyme was sparked when it was found that its proficiency, $k_{cat}/(K_m/k_{non})$, is 2.0×10^{23} M^{-1}, making

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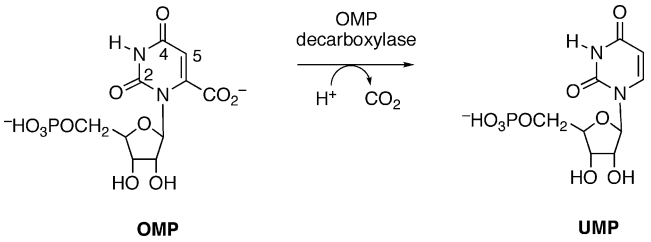
SCHEME 1



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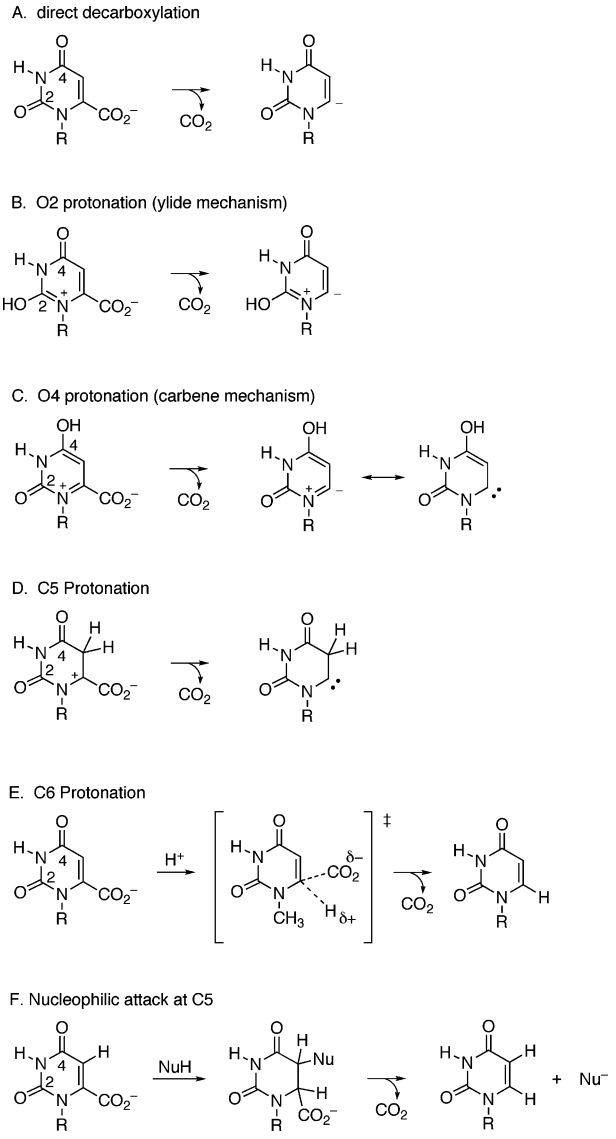

ODCase one of the most proficient enzymes known.[3] A higher proficiency indicates that an enzyme will be more susceptible to transition-state analogues as inhibitors; knowing the enzyme mechanism could thus lead to improved design of inhibitors as antitumor and antiparasite drugs.

Several mechanisms for the decarboxylation of OMP have been proposed.[1,2,4] Prevalent among them is proton transfer to the 2-oxygen (the "ylide" mechanism, Scheme 2B) or to the 4-oxygen (the "carbene" mechanism, Scheme 2C), proposed by Beak and co-workers, and Lee and Houk, respectively.[7,8] The ylide mechanism has been shown by calculations to be highly energetically favorable as compared to the uncatalyzed, "direct" decarboxylation (Scheme 2A).[8] The carbene mechanism (Scheme 2C), involving decarboxylation of the 4-oxygen protonated zwitterion to form a stabilized carbene, has been calculated to be slightly more favorable than the ylide pathway.[8,9]

In 2000, crystal structures of free and bound ODCase from four different species were independently published by the groups of Ealick and Begley, Short and Wolfenden, Larsen, and Pai and Gao.[10−13] Examination of these crystal structures led to a third catalytic proposal involving direct decarboxylation without proton transfer; the main proposal involves catalysis through ground-state electrostatic destabilization. This ground-state destabilization hypothesis has led to further debate and additional studies probing this mechanistic hypothesis.[1,2,4,14−16]

Other proposed mechanisms include proton transfer to the C5 site followed by decarboxylation (Scheme 2D) and a direct protonation-at-C6/decarboxylation mechanism (Scheme 2E).[10,17] A covalent mechanism involving nucleophilic attack at C5 (Scheme 2F) has also been proposed, but this was subsequently shown by ^{13}C and D isotope effects to be unlikely.[18−20]

Numerous studies ranging from the crystal structures to mutagenesis have been undertaken in the past few decades, particularly within the last six years, to elucidate the mechanism.[1,2,4,21,22] The use of analogues of OMP can be particularly valuable in mechanistic studies.[1,2,4,7−9,21,23−27] Noteworthy among analogue studies is the attempt to elucidate the importance of the carbonyl groups by substituting sulfur for oxygen on the orotate ring.[23,27,28]

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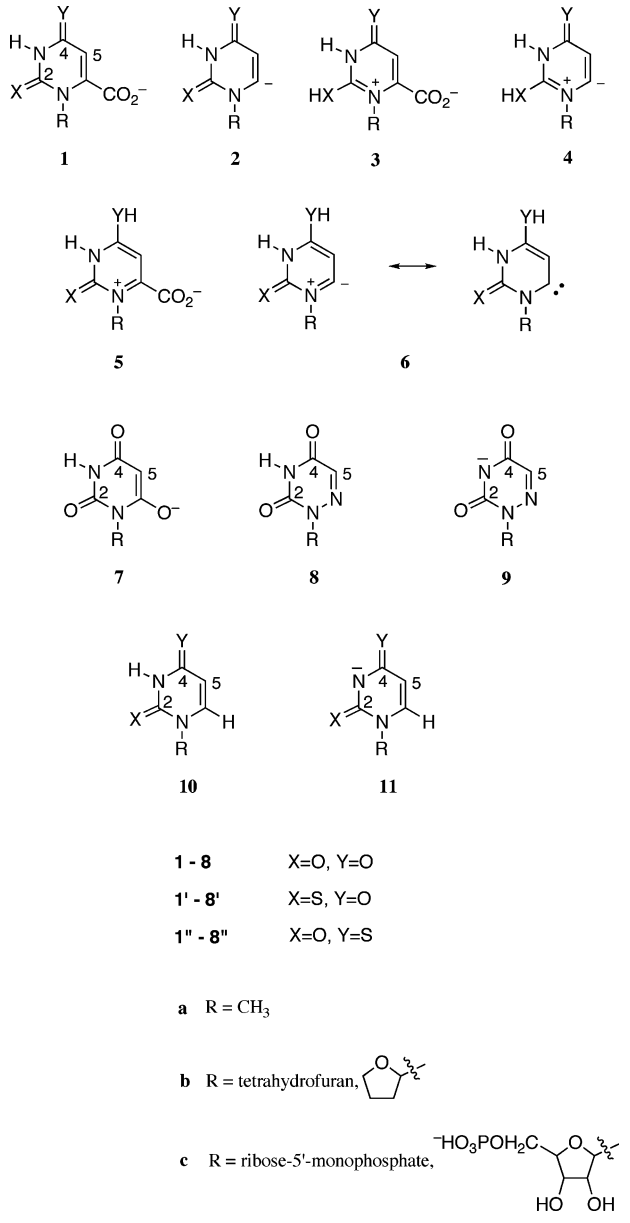
Shostak and Jones examined the decarboxylations of 2-thio-orotidine 5′-monophosphate (2-thio-OMP; **1c′**) and 4-thio-orotidine 5′-monophosphate (4-thio-OMP; **1c″**).[28] They found that the decarboxylation of 4-thio-OMP (**1c″**) occurs in the presence of ODCase at a rate constant of about half that of OMP (**1c**) [k_{cat}(**1c″**) = 9 s^{-1}; k_{cat}(**1c**) = 19 s^{-1}]. On the other hand, they observed *no* catalytic activity of ODCase toward 2-thio-OMP [k_{cat}(**1c′**) not detected]. 2-Thio-OMP is also not a very good inhibitor; its K_m is more than an order of magnitude higher than that of natural OMP [K_m(**1c′**) = 29 μM; K_m(**1c**) = 1.5 μM]. Since 4-thio-OMP **(1c″)** appears to be a viable substrate, while 2-thio-OMP **(1c′)** does not, Shostak and Jones hypothesized that the O2 site plays a critical role in the catalyzed decarboxylation reaction.



A decade after the publication of Jones' work, Smiley and co-workers further studied the activity of ODCase in the presence of 2-thio-OMP (**1c′**).[23] Their results are generally in agreement with those of Shostak and Jones: 2-thio-OMP shows poor binding ($K_m \gg 100$ μm) and a negligible k_{cat}. These authors also propose that 2-thio-OMP may not be able to bind in the enzyme due to the bulky sulfur at the 2-position.

Why is 2-thio-OMP a poor substrate while 4-thio-OMP is relatively good? Herein, we investigate the effect and implications of 2- and 4-thio substitution from a theoretical perspective, focusing on the effect of the sulfur on the direct decarboxylation, ylide, and carbene mechanisms, as well as on the preferred conformations of ODCase substrates.

Theoretical Methods

All calculations were conducted using Gaussian98 and Gaussian03 at 298.15 K.[29,30] Full optimizations of geometries for thio-substituted 1-methyl-orotate **1a′** and **1a″**, thio-substituted deprotonated 1-methyl-uracil **2a′** and **2a″**, 2-protonated thio-substituted 1-methyl-orotate **3a′** and **3a″**, 2-protonated thio-substituted deprotonated 1-methyl-uracil **4a′** and **4a″**, 4-protonated thio-substituted 1-methyl-orotate **5a′** and **5a″**, and 4-protonated thio-substituted deprotonated 1-methyl-uracil **6a′** and **6a″** were determined at the B3LYP/6-31+G* level. B3LYP methods have been previously shown to provide reliable relative energetics for decarboxylations.[8,9,31,32] Zero point energies are included and are unscaled.

The effect of the nucleobase rotation about the N1−C1′ glycosidic bond on overall substrate energy was calculated for OMP analogues where the N1−R group is tetrahydrofuran (THF) to mimic the ribose phosphate. Energies of the structures resulting from rotation of the nucleobase about the glycosidic bond (N1−C1′) in 10° dihedral (torsion) increments were determined at the RHF/6-31+G* level. These structures were all fully optimized except for the fixed torsion angle. This level was chosen as a balance between accuracy and affordability. Previous calculations with ODCase substrates indicate that the RHF/6-31+G* method provides reliable relative energetics.[8,33] Zero point energies are not included for the rotation studies because the majority of the structures are not

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TABLE 1. ΔE for the Decarboxylation of Substrates 1a, 3a, 5a, 1a′, 3a′, 5a′, 1a″, 3a″, and 5a″ at the B3LYP/6-31+G* Level (kcal mol^{-1})[a]

	reaction	ΔE (kcal mol^{-1})
unsubstituted:[b]	**1a** → **2a** + CO_2	35.0
	3a → **4a** + CO_2	11.8
	5a → **6a** + CO_2	11.3
2-thio:	**1a′** → **2a′** + CO_2	31.3
	3a′ → **4a′** + CO_2	10.7
	5a′ → **6a′** + CO_2	9.8
4-thio:	**1a″** → **2a″** + CO_2	31.8
	3a″ → **4a″** + CO_2	11.2
	5a″ → **6a″** + CO_2	11.0

[a] Zero point energies are included. [b] Reference 9.

full minima. The starting structure used for the rotation calculations was the crystal structure for uridine 5′-monophosphate bound to ODCase in *Bacillus subtilis*, in which the O4′−C1′−N1−C2 dihedral angle in the crystal structure is 69°.[10] In none of the four crystal structures do hydroxyl groups on the ribose moiety appear to be in close proximity to the nucleobase, so the use of a THF model in this respect should be acceptable.

Rotation data were analyzed by plotting energies as a Boltzmann distribution. The probability of occurrence of a structure with energy E is described by a Boltzmann distribution, $e^{-E/RT}/q$, where q is the sum of probabilities for all structures.

Single point energy determinations of rotational conformers in the presence of water were conducted at the RHF/6-31+G* level using the CPCM polarizable conductor calculation model.[34,35] Electrostatic potentials were calculated at the B3LYP/6-31+G* level using Gaussian03; figures were generated with GaussView 3.0 (isodensity setting 0.0004 and electrostatic potential range ±0.16 au).[30,36]

Results

A. Energetics. We first explored the energetics of the decarboxylation of both 2-thio-1-methyl-orotate (**1a′**) and 4-thio-1-methyl-orotate **(1a″)**. In probing these energetics, we focused on mechanisms A through C in Scheme 2, that is, the effect of sulfur substitution on direct decarboxylation, on the ylide mechanism, and on the carbene mechanism. These three mechanisms are prevalent among those proposed, and the two involving proton transfer are the most likely to be affected by sulfur substitution.[37−39] Because 2-thio-OMP appears, experimentally, to be a poor substrate, while 4-thio-OMP is a good substrate, we wanted to see if the calculations would reveal a larger barrier for the 2-thio-OMP analogue decarboxylating along one of the proposed proton-transfer catalytic pathways.

1. 2-Thio-1-methyl-orotate (1a′, 3a′, 5a′). Decarboxylation of 2-thio-1-methyl-orotate (**1a′**) is highly endothermic; ΔE = 31.3 kcal mol^{-1} (Table 1). This value is only slightly less than the ΔE calculated for the decarboxylation of the parent 1-methyl-orotate (**1a)** (Table 1). 2-Protonated 2-thio-1-methyl-orotate (**3a′**) is predicted to decarboxylate with a substantially lower ΔE of 10.7 kcal mol^{-1}. Protonation of the 4-oxygen of the 2-thio substrate **5a′** results in an even more favorable reaction; ΔE = 9.8 kcal mol^{-1}. These values are not too dissimilar from the ΔE's calculated for the parent 2- and 4-protonated 1-methyl-orotates (**3a** and **5a;** Table 1).[9]

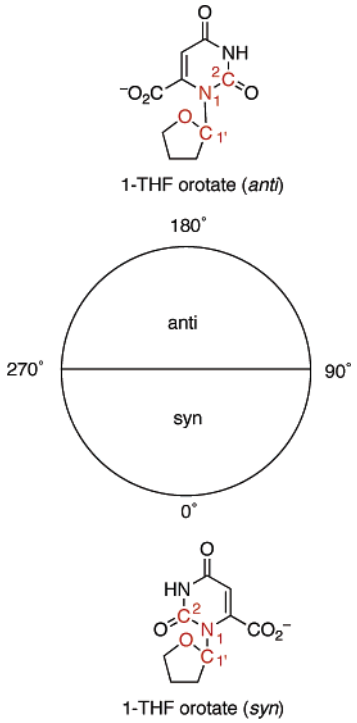

FIGURE 1. Syn versus anti orientation. Glycosidic torsion angle is O4′−C1′−N1−C2.

2. 4-Thio-1-methyl-orotate (1a″, 3a″, 5a″). We find that 4-thio-1-methyl-orotate (**1a″**) has a substantial barrier to decarboxylation; ΔE = 31.8 kcal mol^{-1} (Table 1). Protonation of this substrate at the 2-oxygen (**3a″**) decreases the ΔE to 11.2 kcal mol^{-1}. Protonation at the 4-sulfur (**5a″**) also decreases the energy required for the reaction significantly; ΔE = 11.0 kcal mol^{-1}.

B. Rotation Studies. To fully explore the effects of replacing oxygen with sulfur, we investigated the relationship between the orientation of the nucleobase (relative to the sugar) and the energy of the substrate. In the published crystal structures of bound ODCase, the four inhibitors are observed only in the syn conformation, where syn is defined as an O4′−C1′−N1−C2 torsion angle of 90° through 0° to 270° and anti is defined as an O4′−C1′−N1−C2 torsion angle of 90° through 180° to 270° (Figure 1).[10−13,40] To model the phosphoribosyl moiety for quantum mechanical calculations, we used a tetrahydrofuran (THF) ring as shown in Figure 1.

The energies and Boltzmann probabilities of rotational conformers were calculated for a series of ODCase substrates.The examined substrates are as follows: the 1-THF derivatives of the parent and thio-substituted analogues of the reactant orotate (Figure 2); the initial decarboxylation product uracil C6 anion (Figure 3); the

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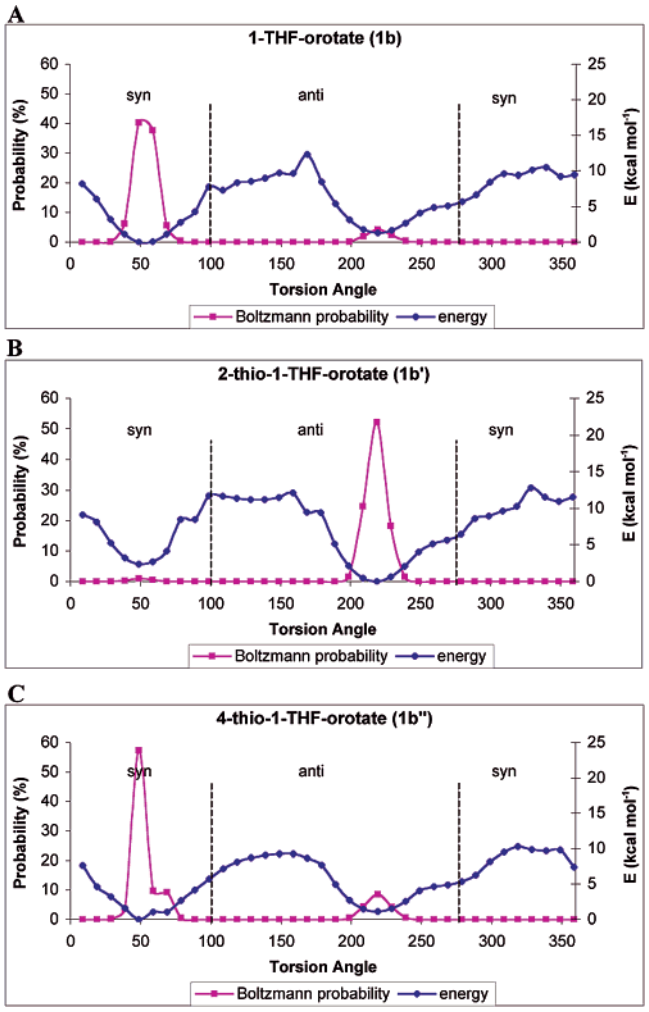

FIGURE 2. Boltzmann probabilities and relative energies of conformers plotted as a function of the glycosidic torsion angle for 1-THF-orotate (**1b**), 2-thio-1-THF-orotate (**1b′**), and 4-thio-1-THF-orotate (**1b″**).

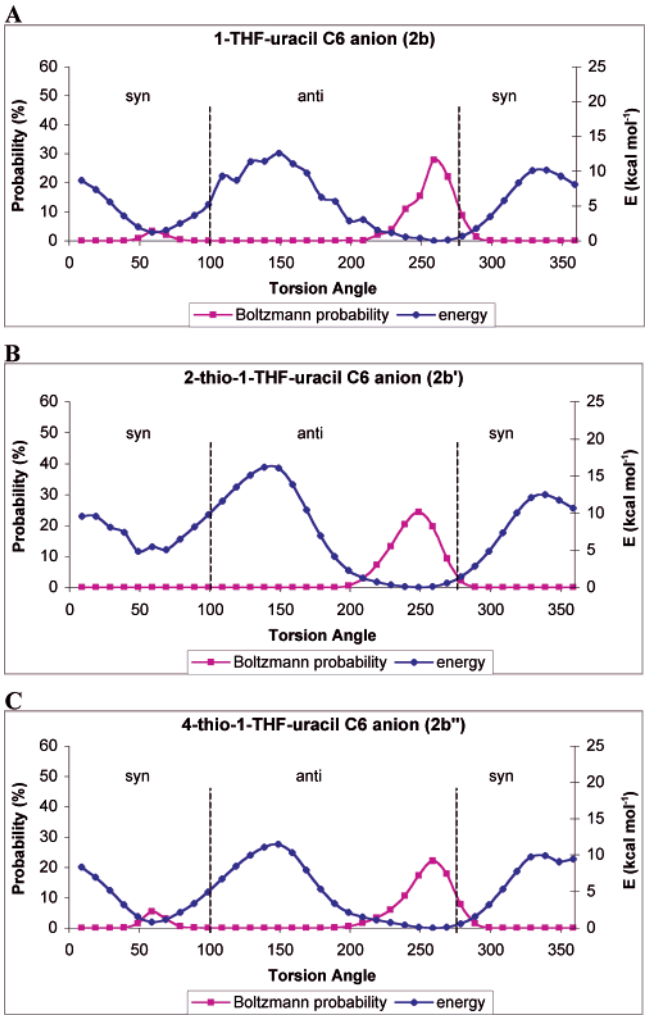

FIGURE 3. Boltzmann probabilities and relative energies of conformers plotted as a function of the glycosidic torsion angle for 1-THF-uracil C6 anion (**2b**), 2-thio-1-THF-uracil C6 anion (**2b′**), and 4-thio-1-THF-uracil C6 anion (**2b″**).

final, neutral decarboxylation product uracil (Figure 5); and uracil N3 anion, a proposed ODCase inhibitor (Figure 6). We also examined the 1-THF derivatives of the ODCase inhibitors barbiturate 5′-monophosphate (BMP), 6-azaUMP, and 6-azaUMP anion (Figure 4). The probabilities and relative energies (energies relative to the lowest energy conformation for each species) were plotted versus the torsion angles. One general feature that we found in all the plots was the presence of two *energy* minima (*probability* maxima). One energy minimum is always found in the syn region, while the other resides in the anti region. The lower energy minimum will be referred to as the favored conformer; the higher energy minimum will be referred to as the disfavored conformer. For some species, the lowest energy minimum is syn; for others it is anti. Energy differences between favored and disfavored conformers are reported as the difference (syn − anti), where syn is the lowest energy structure found in the syn region and anti is the lowest energy structure found in the anti region. Distinct energy maxima exist "between" the favored and disfavored conformers as one rotates from 0° to 360°, suggesting a resistance to rotation between the favored and disfavored conformers. The energy maxima are reported as the lowest barrier that allows for rotation from the favored to the disfavored conformer.

1. 1-THF-orotate (1b), 2-thio-1-THF-orotate (1b′), and 4-thio-1-THF-orotate (1b″) (Figure 2). Boltzmann probability and energy calculations indicate that 1-THF-orotate (**1b**; Figure 2A) and 4-thio-1-THF-orotate (**1b″**; Figure 2C) prefer a syn conformation, with the highest probability of the syn conformer occurring between 35° and 70°. The preference for the syn conformation for orotate **1b** is 1.3 kcal mol^{-1} (Table 2). The syn → anti barrier is 10.5 kcal mol^{-1}. For the 4-thio-orotate **1b″**, the preference for the syn conformation is 1.1 kcal mol^{-1} and the rotation barrier to the anti conformation is 9.3 kcal mol^{-1}. In contrast to **1b** and **1b″**, 2-thio-1-THF-orotate (**1b′**) shows a preference for the anti conformer. The anti conformer is preferred over the syn by 2.4 kcal mol^{-1}, and the lowest rotation barrier between the two conformers (anti → syn) is 12.1 kcal mol^{-1}. The calculated structures for the two minima and the two maxima for **1b**, **1b′**, and **1b″** are shown in Figure 7.

As a confirmation that the RHF/6-31+G* level is a reasonable method for these studies, we also mapped the conformational profile for **1b**, **1b′**, and **1b″** at the B3LYP/

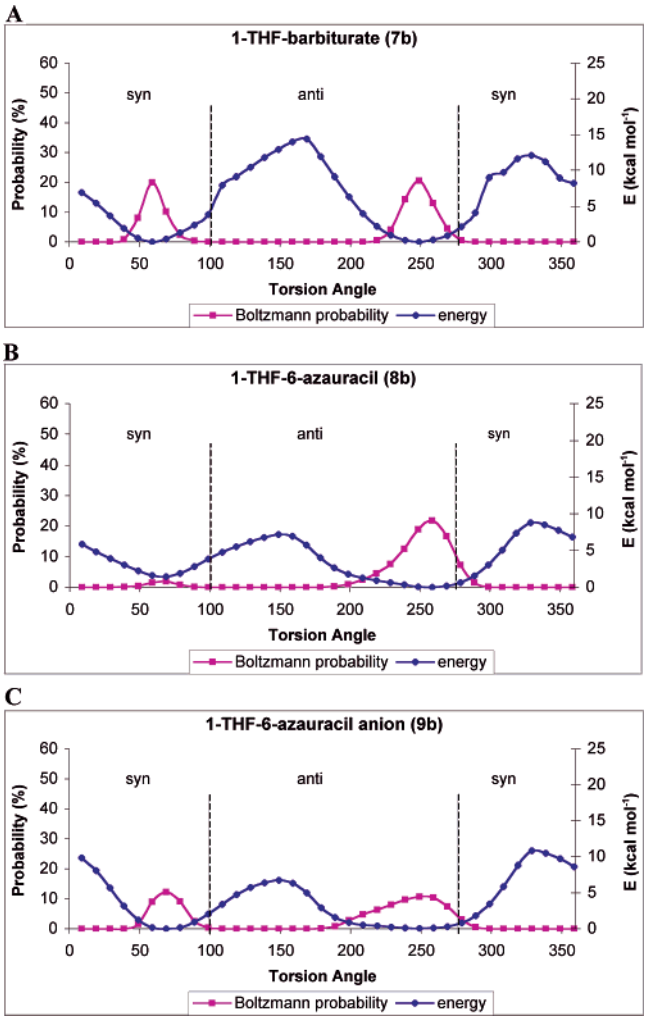

FIGURE 4. Boltzmann probabilities and relative energies of conformers plotted as a function of the glycosidic torsion angle for 1-THF-barbiturate (**7b**), 1-THF-6-azauracil (**8b**), and 1-THF-6-azauracil anion (**9b**).

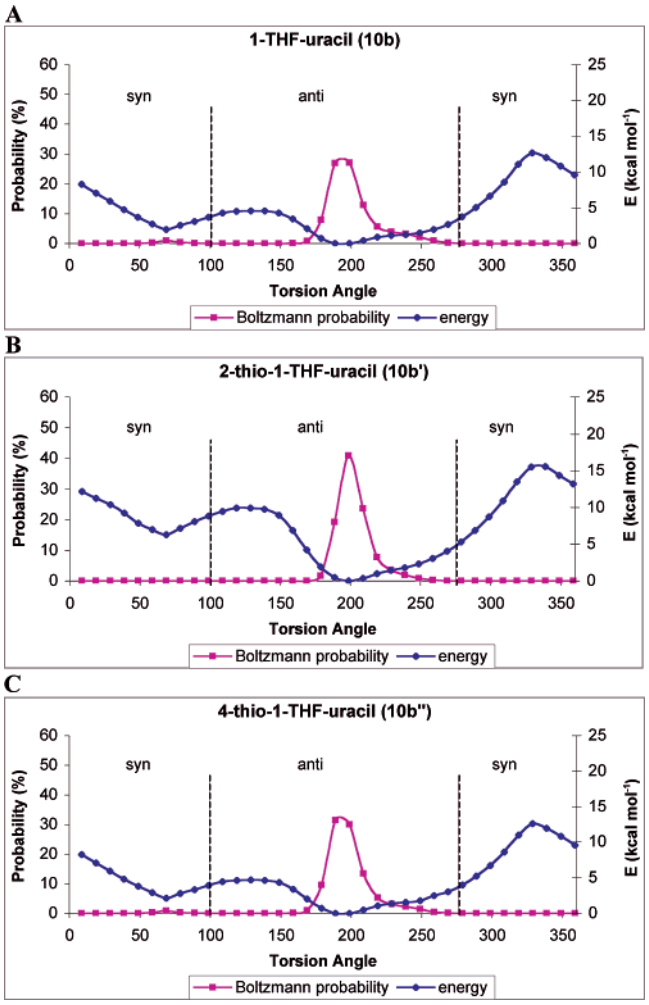

FIGURE 5. Boltzmann probabilities and relative energies of conformers plotted as a function of the glycosidic torsion angle for 1-THF-uracil (**10b**), 2-thio-1-THF-uracil (**10b′**), and 4-thio-1-THF-uracil (**10b″**).

6-31+G* level.[41] The same preferences are found using B3LYP as are found using RHF; while **1b** and **1b″** favor a syn conformation (by 1.3 and 1.1 kcal mol^{-1}, respectively, equivalent to the values found at the RHF/6-31+G* level), 2-thio-1-THF-orotate (**1b′**) favors an anti conformation (by 2.4 kcal mol^{-1}, comparable to the RHF value of 2.7 kcal mol^{-1}). We also conducted *full* optimizations at both RHF/6-31+G* and B3LYP/6-31+G* levels of the minima to check the energies in Table 2. The values obtained from these full optimizations of the minima are consistent with the RHF/6-31+G* partially optimized values (Table 3). MP2/6-31+G* single points on the fully optimized B3LYP/6-31+G* geometries were also conducted as a final check of the energies: for **1b** and **1b″**, the syn conformation is still preferred though by slightly less (0.8 and 0.6 kcal mol^{-1}, respectively); the **1b′** preference for the anti conformation is still clear (syn − anti = 3.1 kcal mol^{-1}). We therefore conclude that the general trends we see using the partially optimized (i.e., fully optimized except for the torsion angle) RHF/6-31+G* structures are reasonable.

(41) The conformational profiles for **1b**, **1b′**, and **1b″** at the B3LYP/6-31+G* level can be found in the Supporting Information.

To ascertain whether the conformational preferences calculated in vacuo would change in solvent, we calculated the energy of the most stable and least stable syn and anti conformers for each species in a dielectric medium of 78.4, that of water (Table 4). The (syn − anti) value for 1-THF-orotate (**1b**) is −0.5 kcal mol^{-1} in water versus −1.3 kcal mol^{-1} in the gas phase. That is, the syn conformer is still favored, but by slightly less. The barrier in water for rotation between syn and anti (syn → anti) is 5.5 kcal mol^{-1} versus 10.5 kcal mol^{-1} in the gas phase.

The (syn − anti) value for 2-thio-1-THF-orotate (**1b′**) is 1.7 kcal mol^{-1} in water versus 2.4 kcal mol^{-1} in the gas phase. In this case, the anti conformer is favored, as it is in vacuo, but again by slightly less. The barrier in water for rotation between anti and syn (anti → syn) is 5.8 kcal mol^{-1}, versus 12.1 kcal mol^{-1} for rotation in the gas phase.

The (syn − anti) value for 4-thio-1-THF-orotate (**1b″**) is −0.3 kcal mol^{-1} in water versus −1.1 kcal mol^{-1} in the gas phase. As for 1-THF-orotate (**1b**), the syn conformer is favored for 4-thio-1-THF-orotate (**1b″**), again by slightly less than it is in the gas phase. The barrier in water for rotation between syn and anti (syn → anti) is 3.7 kcal mol^{-1}, versus 9.3 kcal mol^{-1} for rotation in the gas phase.

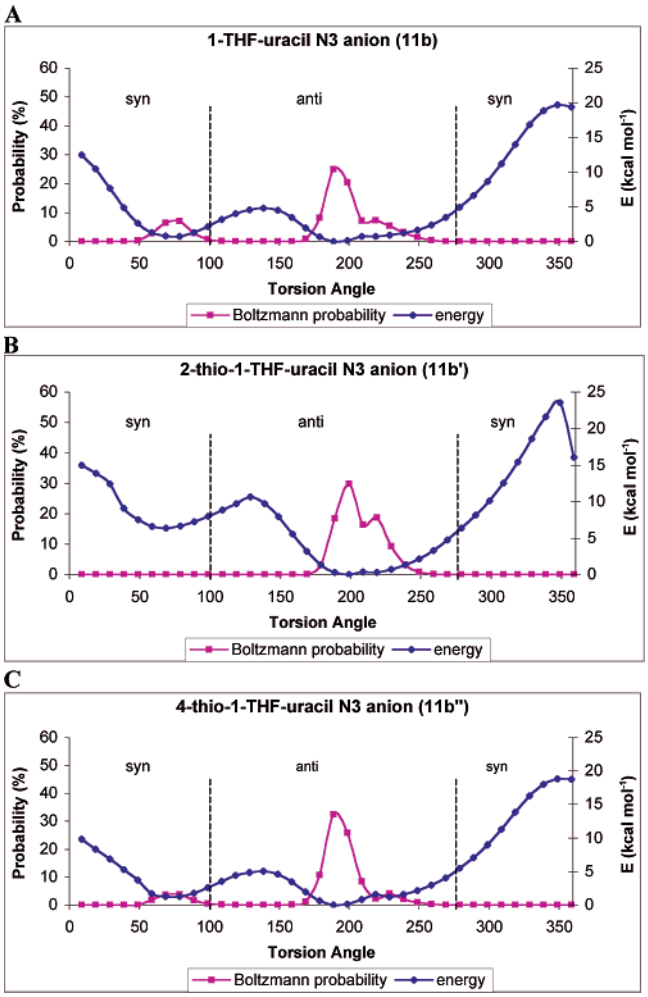

FIGURE 6. Boltzmann probabilities and relative energies of conformers plotted as a function of the glycosidic torsion angle for 1-THF-uracil N3 anion (**11b**), 2-thio-1-THF-uracil N3 anion (**11b′**), and 4-thio-1-THF-uracil N3 anion (**11b″**).

2. 1-THF-uracil C6 Anion (2b), 2-thio-1-THF-uracil C6 Anion (2b′), and 4-thio-1-THF-uracil C6 Anion (2b″) (Figure 3). 1-THF-uracil C6 anion (**2b**), 2-thio-1-THF-uracil C6 anion (**2b′**), and 4-thio-1-THF-uracil C6 anion (**2b″**) all exhibit a preference for the anti conformation. Boltzmann distributions indicate that there is a small probability that the 1-THF-uracil C6 anion (**2b**) exists in the syn conformation. The same holds true for 4-thio-1-THF-uracil C6 anion (**2b″**). Interestingly, the probability of 2-thio-1-THF-uracil C6 anion (**2b′**) existing as the syn conformation is virtually zero. The preference for the anti conformation for 1-THF-uracil C6 anion (**2b**) is 1.2 kcal mol^{-1} (Table 2). The barrier from the anti to the syn conformation (anti → syn) is 10.1 kcal mol^{-1}. For the 2-thio-uracil anion **2b′**, the preference for the anti conformation is 4.8 kcal mol^{-1} and the barrier to the syn conformation (anti → syn) is 12.5 kcal mol^{-1}. For the 4-thio-uracil anion **2b″**, the preference for the anti conformation is 0.8 kcal mol^{-1} and the barrier to the syn (anti → syn) conformation is 9.9 kcal mol^{-1}.

3. 1-THF-barbiturate (7b), 1-THF-6-azauracil Neutral (8b), and 1-THF-6-azauracil Anion (9b) (Figure 4). We also examined the THF derivatives of the ODCase inhibitors barbiturate 5′-monophosphate (BMP, **7c**), neutral 6-azauridine 5′-monophosphate (6-azaUMP, **8c**), and deprotonated (anionic) 6-azauridine 5′-monophosphate (**9c**). 1-THF-barbiturate (**7b**) shows fairly equal population distributions for both syn and anti conformations (Figure 4A). Neutral 1-THF-6-azauracil (**8b**) favors an anti conformation, while the anionic form **9b** is fairly evenly distributed between syn and anti conformers. The (syn − anti) value for **7b** is 0.0 kcal mol^{-1}; the rotational barrier is 12.1 kcal mol^{-1} (Table 2). Similarly, there is no conformational preference for the 1-THF-6-azauracil anion (**9b**) (syn − anti = 0.1 kcal mol^{-1}), and the rotational barrier between conformers (syn → anti) is 6.7 kcal mol^{-1}. We do find a preference of 1.4 kcal mol^{-1} for the anti conformation for 1-THF-6-azauracil (**8b**). The barrier from the anti to the syn conformation (anti → syn) is 7.2 kcal mol^{-1}.

4. 1-THF-uracil (10b), 2-thio-1-THF-uracil (10b′), and 4-thio-1-THF-uracil (10b″) (Figure 5). Boltzmann distributions were also calculated for models of neutral UMP and its thio analogues. For 1-THF-uracil (**10b**) and 4-thio-1-THF-uracil (**10b″**), the populations of conformers are almost entirely anti with a very small population of conformers present in the syn conformation. The population distribution for 2-thio-1-THF-uracil (**10b′**) is exclusively anti. The preference for the anti conformation for 1-THF-uracil (**10b**) is 2.0 kcal mol^{-1} (Table 2). The anti → syn barrier is 4.6 kcal mol^{-1}. For 2-thio-uracil (**10b′**), the preference for the anti conformation is 6.3 kcal mol^{-1} and the barrier to the syn conformation (anti → syn) is 9.9 kcal mol^{-1}. For the 4-thio-uracil (**10b″**), the preference for the anti conformation is 2.2 kcal mol^{-1} and the barrier to the syn conformation (anti → syn) is 4.7 kcal mol^{-1}.

5. 1-THF-uracil N3 Anion (11b), 2-thio-1-THF-uracil N3 Anion (11b′), and 4-thio-1-THF-uracil N3 Anion (11b″) (Figure 6). The Boltzmann distributions of 1-THF-uracil N3 anion (**11b**) and 4-thio-1-THF-uracil N3 anion (**11b″**) show a preference for anti conformations, though syn conformers are also present. Calculations indicate that 2-thio-1-THF-uracil N3 anion (**11b′**) will exist entirely in an anti conformation. The preference for the anti conformation for 1-THF-uracil N3 anion (**11b**) is 0.7 kcal mol^{-1} (Table 2). The anti → syn barrier is 4.8 kcal mol^{-1}. For the 2-thio-uracil (**11b′**), the preference for the anti conformation is 6.3 kcal mol^{-1} and the barrier to the syn conformation (anti → syn) is 10.6 kcal mol^{-1}. For the 4-thio-uracil (**11b″**), the preference for the anti conformation is 1.3 kcal mol^{-1} and the barrier to the syn conformation (anti → syn) is 5.0 kcal mol^{-1}.

Discussion

A. Energetics. 1. Energy Differences. Experimental studies show that ODCase activity is minimal toward 2-thio-OMP; 4-thio-OMP undergoes effective catalysis in the presence of ODCase at about half the rate of OMP.[23,28] The ability of 4-thio-OMP to act as an ODCase substrate could imply that O4 may not play a critical role in the enzymatic decarboxylation reaction, which in turn may imply that the carbene mechanism, which requires O4 protonation, may not be the operative catalytic pathway.[27]

These experimental results prompted us to conduct calculations to investigate how substitution of sulfur for oxygen affects the energetics of three proposed decar-

TABLE 2. Conformational Energy Differences and Rotation Barriers Calculated at the RHF/6-31+G* Level for 1, 2, and 7−11[a]

	preference[b]	(syn − anti) (kcal mol^{-1}) gas phase	favored → disfavored rotation barrier (kcal mol^{-1})
1-THF-orotate **1b**	syn	−1.3	10.5
2-thio-1-THF-orotate **1b′**	anti	2.4	12.1
4-thio-1-THF-orotate **1b″**	syn	−1.1	9.3
1-THF-uracil C6 anion **2b**	anti	1.2	10.1
2-thio-1-THF-uracil C6 anion **2b′**	anti	4.8	12.5
4-thio-1-THF-uracil C6 anion **2b″**	anti	0.8	9.9
1-THF-barbiturate **7b**	none	0.0	12.1
1-THF-6-azauracil **8b**	anti	1.4	7.2
1-THF-6-azauracil anion **9b**	none	0.1	6.7
1-THF-uracil **10b**	anti	2.0	4.6
2-thio-1-THF-uracil **10b′**	anti	6.3	9.9
4-thio-1-THF-uracil **10b″**	anti	2.2	4.7
1-THF-uracil N3 anion **11b**	anti	0.7	4.8
2-thio-1-THF-uracil N3 anion **11b′**	anti	6.3	10.6
4-thio-1-THF-uracil N3 anion **11b″**	anti	1.3	5.0

[a] The only parameter that is constrained is the torsion angle (see text). [b] A preference of "none" indicates the difference between syn and anti conformers is <0.5 kcal mol^{-1}.

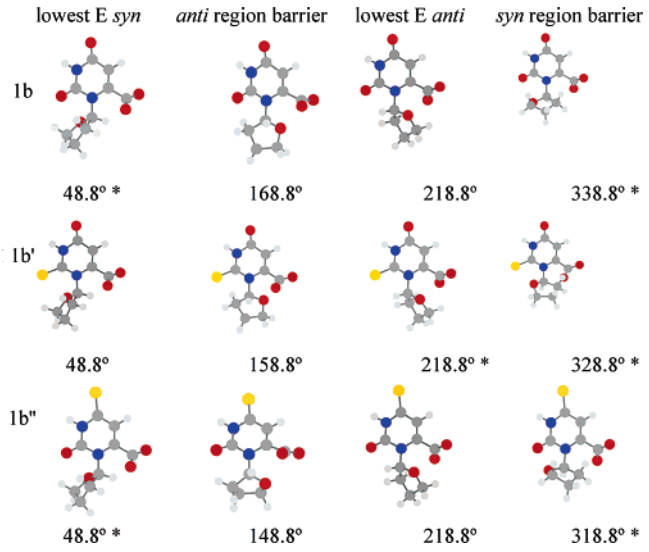

FIGURE 7. Structures corresponding to the energy minima and maxima for **1b**, **1b′**, and **1b″**. For the minima, the asterisk indicates the lower energy minimum. For the maxima, the asterisk indicates the lower energy maximum.

TABLE 3. Comparison of Conformational Energy Differences in the Gas Phase at the RHF/6-31+G* (Partially Optimized Minima), RHF/6-31+G* (Fully Optimized Minima), and B3LYP/6-31+G* (Fully Optimized Minima) Levels for 1b, 1b′, and 1b″

	RHF/6-31+G*, partially optimized[a]	RHF/6-31+G*, fully optimized	B3LYP/6-31+G*, fully optimized
1-THF-orotate **1b**	−1.3	−1.4	−1.3
2-thio-1-THF-orotate **1b′**	2.4	2.4	2.7
4-thio-1-THF-orotate **1b″**	−1.1	−1.2	−1.1

[a] The only parameter that is constrained is the torsion angle (see text).

boxylation mechanisms: the direct decarboxylation (Scheme 2A), the ylide mechanism (Scheme 2B), and the carbene mechanism (Scheme 2C). Our computational studies indicate a clear preference for decarboxylation via

TABLE 4. Comparison of Conformational Energy Differences and Rotation Barriers in the Gas Phase versus in Water for 1b, 1b′, and 1b″; Calculated at the RHF/6-31+G* Level

		(syn−anti) (kcal mol^{-1})	favored → disfavored rotation barrier (kcal mol^{-1})
1-THF-orotate (**1b**)	water	−0.5	5.5
	gas	−1.3	10.5
2-thio-1-THF-orotate (**1b′**)	water	1.7	5.8
	gas	2.4	12.1
4-thio-1-THF-orotate (**1b″**)	water	−0.3	3.7
	gas	−1.1	9.3

protonation as opposed to direct decarboxylation for both 2- and 4-thio-1-methyl-orotate (Table 1). Previous theoretical studies reveal the same preference for the unsubstituted analogue (Table 1).[8,9,25] While the preference for 4-protonation in the 4-thio case is slight at best (11.0 vs 11.2 kcal mol^{-1}), there may be a small preference for 4-protonation when sulfur substitution is at the 2-position (9.8 vs 10.7 kcal mol^{-1}).

These results indicate that protonating the 2- or 4-site, regardless of whether that site is a sulfur or an oxygen, substantially reduces the barrier to decarboxylation. Therefore, neither the ylide nor carbene mechanism can be discounted on the basis of energetics. That is, these calculations show that, should the mechanism involve 2- or 4-protonation, sulfur substitution should not affect the catalysis in terms of energetics; all protonation pathways are energetically favorable (Table 1).

2. Proton Affinities. The component we have not yet considered is the protonation step. In the active site, presumably, a side chain moiety can provide an acidic proton to OMP.[1,2,4] Most likely, the decarboxylation will be the rate-determining step. It is, however, of interest to compare the proton affinities of the parent and sulfur-substituted analogues to ensure that the proton transfer to sulfur is as facile, if not more so, as proton transfer to an oxygen.

Proton affinities (PAs) for the unsubstituted 1-methyl-orotate (**1a**), 2-thio-1-methyl-orotate (**1a′**), and 4-thio-1-methyl-orotate (**1a″**) are depicted in Figure 8. For both the 2- and 4-positions, the thiocarbonyl site is more basic

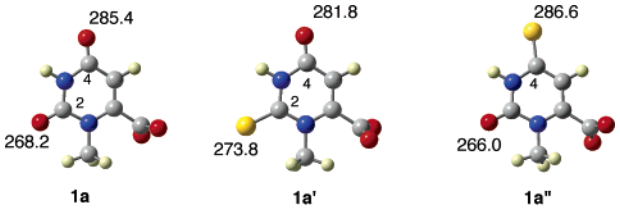

FIGURE 8. B3LYP/6-31+G*-optimized structures of 1-methyl-orotate (**1a**), 2-thio-1-methyl-orotate (**1a′**), and 4-thio-1-methyl-orotate (**1a″**). Proton affinities, in kcal mol^{-1}, are indicated next to each carbonyl and thiocarbonyl.

than the corresponding carbonyl site in the unsubstituted analogue. That is, the 2-oxygen of 1-methyl-orotate **(1a)** has a proton affinity of 268.2 kcal mol^{-1}, while the 2-sulfur of 2-thio-1-methyl-orotate (**1a′**) has a PA of 273.8 kcal mol^{-1}. Likewise, the 4-oxygen of 1-methyl-orotate **(1a)** has a proton affinity of 285.4 kcal mol^{-1}, while the 4-sulfur in the 4-thio-1-methyl-orotate (**1a″**) has a PA of 286.6 kcal mol^{-1}. These values are of interest since they imply that should an initial proton transfer occur for the parent compounds, such a proton transfer would not be energetically unfavorable for the thio analogues, since the thio-PAs are higher than the PAs of the parent compounds.

It is also of interest that the 4-position for each structure is more basic for both the unsubstituted and thio-substituted 1-methyl-orotates, regardless of whether a sulfur or an oxygen is at the 4-position (Figure 8). The C4 carbonyl is more basic than the C2 carbonyl by 17.2 kcal mol^{-1} in the unsubstituted species **1a**, the C4 carbonyl is more basic than the S2 by 8.0 kcal mol^{-1} in the 2-protonated species **1a′**, and the S4 is more basic than the O2 by 20.6 kcal mol^{-1} in the 4-protonated species **1a″**. If catalysis of OMP, 2-thio-OMP, or 4-thio-OMP proceeds via a proton-transfer mechanism, the most favorable site for protonation is the O4/S4.

In essence, our energetics calculations indicate that protonation at either the 2- or 4-position of both thio-substituted substrates should result in a lowered barrier for decarboxylation, with a slight preference for 4-protonation. Therefore, these energetics calculations do not alone explain why 2-thio-OMP does not undergo catalysis.

B. Rotation Studies. Since the calculated energetics do not appear to explain the experimentally observed effects of thio substitution, we sought to calculate the effect of the sulfur on the conformations of ODCase substrates.

1. Conformational Preference (Figures 2−6). Our calculations indicate that, among all the substrates we studied, the syn conformation is preferred only by 1-THF-orotate (**1b**) and 4-thio-1-THF-orotate (**1b″**). We also find that 1-THF-barbiturate (**7b)** and anionic 1-THF-6-aza-uracil (**9b)** show no syn/anti preference; that is, the population ratio of syn/anti is roughly 1:1. All other orotate and uracil structures studied, thio-substituted and not, have an energetic preference for the anti conformation.

Several authors have reported the crystal structures for uridine, 2-thiouridine, and 4-thiouridines as anti.[42−44] Saenger and Scheit found the crystal structure for 4-thiouridine sesquihydrate to be syn but hypothesized that this conformation may be forced by the inclusion of a water of hydration in the crystal lattice.[45] Solution NMR studies indicate that the conformation of aqueous orotidine is syn, while that of uridine is anti.[46] Additionally, the crystal structures of both 6-azauridine and 6-azauridine 5′-phosphate trihydrate show an anti configuration.[47,48] In general, the free pyrimidine nucleosides favor the anti conformation.[40] Our calculations mirror these experimental results in that 1-THF-uracil (**10b**) and its thio-substituted analogues (**10b′**, **10b″**) all prefer the anti conformation. 1-THF-orotate (**1b**) prefers the syn conformation, in agreement with the NMR solution data. 1-THF-6-azauracil (**8b**) prefers the anti conformation, consistent with the crystal structures.

The crystal structures of three inhibitors (UMP (**10c**), 6-azaUMP (**8c**), and BMP (**7c**)) bound to ODCase have been solved, and contrary to observations of the conformations of the free uridine and azauridine nucleosides, these three inhibitors are in the syn conformation when bound in ODCase.[10−13] In the crystal structure of ODCase from *Bacillus subtilis* complexed with UMP (PDB ID code 1dbt), UMP has a syn glycosidic torsion angle of 65.8°.[10] ODCase from *Methanobacterium thermoautotrophicum* complexed with 6-azaUMP (PDB ID code 1dvj) has a syn glycosidic angle of 68.2°.[13] The crystal structures of two other species, *E. coli* (PDB ID code 1eix) and *Saccharomyces cerevisiae* (PDB ID code 1dqx), were cocrystallized with the inhibitor BMP.[11,12] In both cases, the inhibitor is syn with glycosidic angles of 65.2° and 55.3°, respectively.[49] We next considered whether for a given substrate the most stable calculated conformation in some way correlates with that particular substrate's activity.

2. Correlation between Conformational Preference and ODCase Activity. By our calculations, both 1-THF-orotate (**1b)** and 4-thio-1-THF-orotate (**1b″**) prefer a syn conformation, while 2-thio-1-THF-orotate (**1b′**) prefers an anti conformation (Figure 2). ODCase-bound inhibitors are all syn in the crystal structures.[10−13] It seems of interest to us that OMP (**1c**) and 4-thio-OMP (**1c″**) are found to be good substrates experimentally and that our calculations indicate that both prefer a syn conformation. 2-Thio-OMP (**1c′**) is experimentally found to be a poor substrate and by our calculations prefers an anti conformation. Could there be a relationship between conformational preference and the ability to bind to ODCase? Perhaps pyrimidine substrates must be syn either to bind properly and/or to undergo decarboxylation. Although the rotation barriers in Figure 2 are too low to prohibit access to all the rotational conformers at room temperature and our dielectric calculations indicate that a polar medium may in fact decrease those barriers

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(49) Three monomers are present in the crystal structure of ODCase from *Bacillus subtilis* complexed with UMP (PDB ID code 1dbt); four monomers are present in the crystal structures of 1dvj, 1eix, and 1dqx. Each reported glycosidic torsion angle is an average of the torsion angles for all the monomers in each crystal structure.

(Table 4), we still find the trends of interest. OMP (**1c**) and 4-thio-OMP (**1c**′′) are substrates for ODCase, and we find that both, when free "THF nucleosides", prefer syn conformations. 2-Thio-OMP (**1c**′) is not a good ODCase substrate and prefers an anti conformation by calculation. Perhaps 2-thio-OMP binds to ODCase anti, which is not favorable and results in a low K_m; furthermore, the configuration is such that decarboxylation cannot occur. Alternatively, perhaps 2-thio-OMP binds syn, but this results in an energetic penalty that affects K_m and k_{cat}.[50]

3. Correlation between Conformational Preference and Inhibition. Intrigued by the correlation of syn preference and substrate viability, we wondered whether the same correlation could be found with known ODCase inhibitors. Does an inhibitor with a higher calculated preference for the syn conformation have an experimentally lower K_i? We do find that 1-THF-barbiturate (Figure 4A) has a much higher syn population than 1-THF-6-azauracil (Figure 4B); BMP is indeed the most prodigious known ODCase inhibitor (K_i(BMP) = 8.8×10^{-12} M; K_i-(6-azaUMP) = 5.1×10^{-7} M).[51] These results correlate nicely with the idea that a higher syn conformation might indicate a better ODCase binder.

We also calculated the deprotonated form of 6-azauracil. Levine, Brody, and Westheimer have proposed that a negatively charged pyrimidine ring is required for binding.[51] This hypothesis is based on the facts that OMP exists as an anion over the range of pH in which ODCase is active and that 6-azaUMP is a more effective inhibitor when the triazine ring is ionized.[51,52] Therefore, Westheimer and co-workers propose that only ionized 6-aza-UMP binds to ODCase.

The conformer population of ionized 1-THF-6-azauracil (**9b**, Figure 4C) shows a fairly equivalent amount of syn and anti conformers. The greater proportion of syn conformers, by our calculational hypothesis, implies that the 1-THF-6-azauracil anion **9b** is a better inhibitor than its neutral protonated counterpart **8b**. This is in agreement with Westheimer's proposal; the apparent K_i for **8c** is 4.6×10^{-8} M while the calculated K_i for **9c** (assuming a pK_a of **8c** of 7.0) is 5.1×10^{-7} M.[51]

Using the same analysis for uracil and its thio analogues (Figure 5), we do not see the expected correlation between a syn preference and inhibition; neutral 6-aza-UMP ($K_i = 5.1 \times 10^{-7}$ M) is a better inhibitor than UMP (two reported values, $K_i = 4.6 \times 10^{-4}$ and 9.2×10^{-5} M), but the percent syn seems similar (Figure 4B versus Figure 5A).[27,51] However, the syn population for 1-THF-uracil and its thio analogues is still either very small or nonexistent as compared with the anti population. None of the charts in Figure 5 even remotely resemble Figure 4A or C; there are no significant populations of the syn conformer in any of the charts in Figure 5. Consistent with our observation that less syn should indicate worse binding, the K_i's for UMP, 2-thio-UMP, and 4-thio-UMP are indicative of poor inhibition (9.2×10^{-5} M, 4.3×10^{-5} M, and 1.5×10^{-6} M, respectively).[27]

(50) The idea that rotational conformers might play a role in ODCase binding has also been suggested by Smiley and co-workers (ref 23), although they speculated that OMP might prefer binding in an anti conformation.

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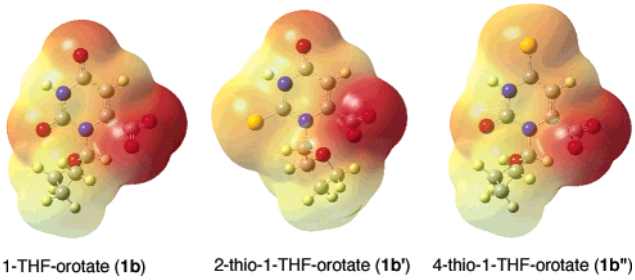

FIGURE 9. B3LYP/6-31+G*-calculated electrostatic potential surface for 1-THF-orotate (**1b**), 2-thio-1-THF-orotate (**1b**′), and 4-thio-1-THF-orotate (**1b**′′). Red indicates negative potential while blue indicates positive potential.

We also considered N3-deprotonated UMP as an inhibitor, to explore the Westheimer proposal that the active binding form of UMP is actually the N3-deprotonated anion.[51] The calculated K_i values for deprotonated UMP, 2-thio-UMP, and 4-thio-UMP are 9.2×10^{-5} M, 4.3×10^{-5} M, and 1.5×10^{-6} M, respectively, assuming respective pK_a's of 9.5, 9.2, and 8.6.[27] If these anions are better inhibitors than the neutral counterparts, by our theory the syn population should be greater. Indeed, for the anions in Figure 6A and C we see a greater syn population than that for their respective neutral counterparts in Figure 5A and C. Our theory, however, breaks down for Figure 6B (2-thio-1-THF-uracil-N3 anion); no syn conformers are present, although the K_i of anionic 2-thio-UMP is similar to that of anionic UMP. Perhaps anionic 2-thio-UMP is not a substrate; the K_i is calculated based on apparent K_i and pK_a values, but there is no proof that the anionic form actually binds.[27,51]

4. Energy Barriers to Rotation. Differences in energies between favored and disfavored conformers range from 0.1 to 6.3 kcal mol^{-1}; *barriers* between favored and disfavored conformers range from 4.8 to 12.5 kcal mol^{-1}. We find that barriers for the 2-thio species tend to be higher than those of the analogous unsubstituted and 4-thio species. For example, while 2-thio-1-THF-orotate (**1b**′) has an anti → syn barrier of 12.1 kcal mol^{-1}, 1-THF-orotate (**1b**) and 4-thio-1-THF-orotate (**1b**′′) have syn → anti barriers of 10.5 and 9.3 kcal mol^{-1}, respectively. The other nucleobases follow this trend (Table 2: **1b**′ vs **1b** and **1b**′′, **2b**′ vs **2b** and **2b**′′, **10b**′ vs **10b** and **10b**′′, **11b**′ vs **11b** and **11b**′′). Presumably, this is due to steric interactions originating from the larger sulfur. Although the barriers are not large enough to inhibit rotation, the trends are still of interest; the 2-sulfur substrates clearly are less dynamic in structure than the parent or 4-sulfur species.

To further probe the effect of substituting sulfur for oxygen, we calculated the molecular electrostatic potential (MEP) of 1-methyl-orotate, 2-thio-1-methyl-orotate, and 4-thio-1-methyl-orotate (Figure 9). The color at each point on these surfaces reflects the interaction energy between the molecule and a positive test charge at that point. Red indicates an attractive potential while blue represents a repulsive potential. The areas of red therefore indicate a "negative" region; yellow/green indicates a more neutral or "positive" region, depending on how bluish the color. In all three structures, the area around the carboxylate is quite red, consistent with the fact that it is negatively charged. The lower electronegativity of

sulfur versus oxygen is also displayed by the MEPs; comparison of the parent 1-methyl-orotate with the thio-substituted analogues shows a decrease in the "redness" of the 2- and 4-positions when substituted with sulfur. The shape of the surfaces, particularly for 2-thio-1-methyl-orotate, is of particular interest. The sulfur appears to be as bulky as the carboxylate; the higher rotation barriers for the 2-thio substrates are therefore not surprising.

Conclusions

Theoretical studies of thio-substituted ODCase substrates have uncovered interesting trends. While sulfur substitution does not appear to affect the energetics of decarboxylation, it does lead to definite preferences for rotational conformation, presumably due to the bulkiness of the sulfur. One tantalizing idea is that how well a substrate binds to ODCase may be dependent on the tendency of the substrate to favor a syn rotational conformation. The next step to further probe these trends is to conduct more complex calculations with the N1 substituted with a phosphoribosyl group and to include solvation; studies are currently underway.

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Supporting Information Available: Cartesian coordinates for all calculated species and a figure showing Boltzmann probabilities and relative energies versus torsion angle in **1b**, **1b′**, and **1b″** calculated at the B3LYP/6-31+G* level. This material is available free of charge via the Internet at http://pubs.acs.org.

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