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Lys314 is a Nucleophile in Non-Classical Reactions of Orotidine-5′-Monophosphate Decarboxylase

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Abstract: Orotidine-5′-monophosphate decarboxylase (OMPD) catalyzes the decarboxylation of orotidine-5′-monophosphate (OMP) to uridine-5′-monophosphate (UMP) in an extremely proficient manner. The reaction does not require any cofactors and proceeds by an unknown mechanism. In addition to decarboxylation, OMPD is able to catalyze other reactions. We show that several C6-substituted UMP derivatives undergo hydrolysis or substitution reactions that depend on a lysine residue (Lys314) in the OMPD active site. 6-Cyano-UMP is converted to UMP, and UMP derivatives with good leaving groups inhibit OMPD by a suicide mechanism in which Lys314 covalently binds to the substrate. These non-classical reactivities of human OMPD were characterized by cocrystallization and freeze-trapping experiments with wild-type OMPD and two active-site mutants by using substrate and inhibitor nucleotides. The structures show that the C6-substituents are not coplanar with the pyrimidine ring. The extent of this substrate distortion is a function of the substituent geometry. Structure-based mechanisms for the reaction of 6-substituted UMP derivatives are extracted in accordance with results from mutagenesis, mass spectrometry, and OMPD enzyme activity. The Lys314-based mechanisms explain the chemodiversity of OMPD, and offer a strategy to design mechanism-based inhibitors that could be used for antineoplastic purposes for example.

Keywords: decarboxylation · enzymes · hydrolysis · nucleophilic substitution · nucleotides

Introduction

Orotidine-5′-monophosphate decarboxylase (OMPD) catalyzes the last step of de novo pyrimidine nucleotide biosynthesis, the decarboxylation of orotidine-5′-monophosphate (OMP, **1**) to uridine-5′-monophosphate (UMP, **2**). OMPD accelerates the decarboxylation reaction by 10^{17}-fold compared to the uncatalyzed reaction, and thus constitutes one of the most proficient enzymes known.[1] Despite a plethora of crystal structures of substrate, product, and inhibitor complexes of OMPD from various organisms and a large body of kinetic evidence, a chemical mechanism of OMP decarboxylation has not been agreed on.[2] The active form of OMPD from bacterial, fungal, and mammalian sources is a homodimeric TIM-barrel without any cofactors or metal ions. Important residues in the active site of OMPD are two aspartic acids and a lysine residue (Figure 1). Comparison of the UMP complexes of OMPD from human *Methanobacterium thermoautotrophicum* and *Plasmodium falciparum* shows that whereas the key active-site residues are conserved, the architecture of the active site and the conformation of the catalytic side-chains can differ markedly (Supporting Information, Figure S0). The active-site residues Asp312 and Lys314 (human OMPD numbering) are of particular interest and have been implicated in exerting electrostatic stress on the substrate and in functioning as a proton donor for an intermediate carbanion, respectively.[3,4]

Because the progress of cancer relies on the rapid synthesis of DNA,[5,6] inhibition of enzymes of de novo nucleotide biosynthesis pathways, such as OMPD, is an attractive objective for the development of antineoplastic drugs. OMPD shows unusual chemodiversity, that is, it converts non-natural nucleotides into **2** or other products by substitution, hydrolysis, and decarboxylation reactions that have no coun-

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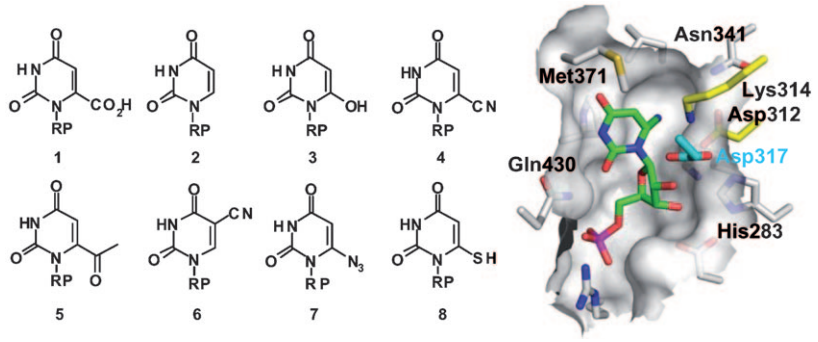


Figure 1. Left: Nucleotides that were investigated as OMPD substrates or inhibitors: OMP (**1**), UMP (**2**), BMP (**3**), 6-cyano-UMP (**4**), 6-acetyl-UMP (**5**), 5-cyano-UMP (**6**), 6-azido-UMP (**7**), 6-mercapto-UMP (**8**). RP denotes the phosphoribosyl residue. Right: The OMPD active-site cavity is shown in gray with its chemical environment shown as stick representation and the nucleotide 6-cyano-UMP (**4**) bound.[3a] Residues in proximity to the nucleobase are labeled. The catalytic residues Lys314 and Asp312 are highlighted in yellow and Asp317 (blue) is contributed from the second subunit.

terpart in vivo. This chemodiversity might be exploited for drug development. OMPD does not tolerate substantial variations at the 5′-phosphoribofuranose moiety, which is required both for high-affinity binding and for substrate conversion.[2a,7] In contrast, OMPD accepts a diverse set of pyrimidine-modified nucleotides, many of which are high-affinity competitive inhibitors, or even exhibit time-dependent covalent inhibition.[5a,8,9] For example, OMPD from *M. thermoautotrophicum* engages in suicide inhibition by nucleophilic aromatic substitution of 6-iodo-UMP by the essential Lys72 (Lys314 in the human enzyme).[7] In addition, *M. thermoautotrophicum* OMPD catalyzes the conversion of 6-cyano-uridine-5′-monophosphate (6-cyano-UMP, **4**) to barbituric acid 5′-monophosphate (BMP, **3**).[10] We found that human OMPD is also covalently inhibited by 6-iodo-UMP, but report here different reaction products for the conversion of **4**.[5a] In humans, **4** is converted to UMP (**2**) and not BMP (**3**); this suggests different reaction mechanisms for the two enzymes.

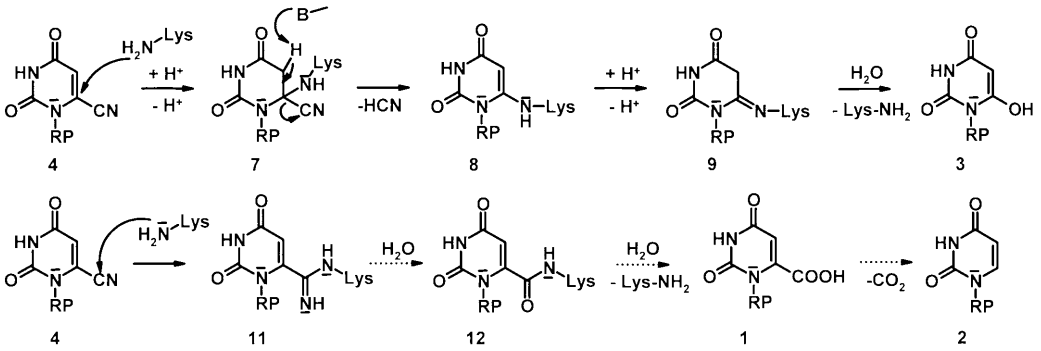
To determine the structural basis for this different behavior and to further probe the determinants for the chemodiversity of OMPD, we synthesized several 5- and 6-substituted UMP derivatives for interaction with wild-type (wt) human OMPD. Two OMPD mutants, Asp312Asn and Lys314Ala, were generated to abolish enzyme activity and trap reaction intermediates. Crystal structures of wt-OMPD that were cocrystallized or briefly soaked with 6-cyano-UMP provided information on the product UMP and a bent reaction intermediate, respectively. Structures of the mutants resulted in unexpected products and revealed that Lys314 exhibits increased nucleophilicity in the Asp312Asn mutant compared to wt-OMPD. Furthermore, the Lys314Ala mutant is still active for hydrolysis and decarboxylation as exemplified by a cocrystal structure with 6-cyano-UMP that resulted in a UMP complex. Understanding the details of such atypical OMPD reactions might give insight into the primary chemical mechanism of OMP decarboxylation.

Results and Discussion

Crystal structures of OMPDs in complex with uridine nucleotides consistently show the *syn* conformation of the ligand; this exposes the C5- and C6-positions of the nucleobase to Lys314. Lys314 is a known key catalytic residue, and ligand design thus focused on C5 and C6-modified UMP nucleotides that could react with this potential nucleophile (Figure 1).[3a,7,11]

Substrate distortion, hydrolysis, and decarboxylation of 6-cyano-UMP: For yeast OMPD 6-cyano-UMP (**4**) was described as a weakly competitive (K_d = 11 μM) and chemically stable inhibitor.[12] Based on the CN stretching frequency, the cyano group was proposed to be slightly bent out of the plane of the pyrimidine ring by approximately 20°.[9] In contrast, 6-cyano-UMP is known to inhibit wt-OMPD from *M. thermoautotrophicum* after its transformation to BMP **3**, which is the most efficient competitive OMPD inhibitor known to date.[8] When human OMPD was cocrystallized with 6-cyano-UMP, the resulting crystal structure surprisingly showed the presence of UMP (**2**) in the active site (data not shown). Within at most a few days 6-cyano-UMP (**4**) was converted to UMP (**2**) by human OMPD and not to BMP (**3**). In both cases, an initial covalent addition of Lys314 can explain the obtained product (Scheme 1).

In principle, involvement of a lysine residue is not necessary. BMP (**3**) could be generated directly from 6-cyano-UMP (**4**) by substitution with water in the *M. thermoautotrophicum* active site. Likewise, **4** could be hydrolyzed to OMP and then decarboxylated by human OMPD. However, the higher nucleophilicity of primary amines compared to water should favor a pathway involving lysine. Also, the 6-cyano-uridine moiety is stable in aqueous solution for several days (unpublished observation). For human OMPD, the likely route is Lys314-assisted hydrolysis of the nitrile **4** to the carboxylic acid OMP **1** via covalent intermediates, followed by normal decarboxylation. The nitrile is thus a masked carboxylic acid, similar to the use of nitriles in organic synthesis. Because hydrolysis of **4** is a prerequisite for UMP (**2**) formation, the active site of human OMPD should allow solvent to enter at later stages during catalysis. The apparent discrepancy in product distribution between the *M. thermoautotrophicum* and human enzymes might be ascribed to species differences of the active sites. For instance, the active-site residues Asn341 and His283 in human OMPD are alanine and glycine, respectively, in *M. thermoautotrophicum* OMPD.[8] These smaller side-chains might change the active-site polarity, leading to a different mecha-



Scheme 1. Covalent mechanisms by using Lys314 as a nucleophile can explain the formation of BMP and UMP from 6-cyano-UMP.

nism, or allow water to more easily enter the active site during catalysis and alter the sequence of reactions.

To evaluate the hypothesis of 6-cyano-UMP hydrolysis prior to decarboxylation and to test if Lys314 acts as a nucleophile in this reaction, two strategies were employed. First, short-time soaks of apo-enzyme crystals with 6-cyano-UMP were performed to trap an enzyme–substrate complex. Second, active-site OMPD mutants that were expected to abolish or reduce activity were generated. We have shown earlier that the human Asp312Asn OMPD variant is inactive with respect to decarboxylation.[3a] In addition, the Lys314Ala mutant lacking the nucleophilic group was generated, thus serving as a negative control. The cocrystal structure of wt human OMPD with 6-cyano-UMP, obtained by a 25 min soak of the substrate with apo-crystals, revealed intact nucleotide bound in the active site (Figure 2). A longer soak of 60 min already showed significant loss of the cyano group; this puts the timescale of hydrolysis–decarboxylation reaction within hours at ambient temperature. In the structure, a water molecule occupies the position of the nitrile nitrogen atom (Figure 2). For reasons of conjugation, the cyano group in cyano-UMP nucleotides **4** and **6** is expected to be coplanar with the aromatic pyrimidine ring. By contrast, in complex with OMPD the 6-cyano group is bent out of the ring plane by 57°, which is much more than anticipated from the CN stretching frequency.[9] Similar substrate distortion has been observed for OMP in the crystal structures of inactivated human and *M. thermoautotrophicum* OMPDs in which the carboxylate groups are also kinked from the pyrimidine ring planes.[3,8]

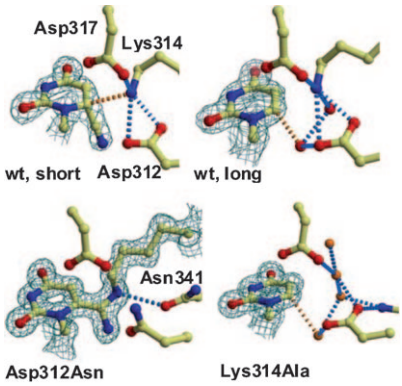


Figure 2. Cocrystal structures of the reaction products of 6-cyano-UMP with OMPD. Top left: wt-OMPD leading to substrate distortion. Top right: wt-OMPD with UMP that was formed from 6-cyano-UMP. Bottom left: OMPD Asp312Asn reacts with 6-cyano-UMP to form a covalent amidine complex. Bottom right: OMPD Lys314Ala degrades 6-cyano-UMP to UMP. Possible hydrogen bonds (<3.2 Å) are shown as dashed blue lines, and unfavorable contacts are highlighted with dashed orange lines. Note the water-mediated hydrogen bond network in the Lys314Ala mutant.

Quite surprisingly, in the Asp312Asn mutant 6-cyano-UMP forms a covalent OMPD–substrate complex. Lys314 is linked to the cyano moiety through an amidine or amide (Figure 2). MALDI-TOF spectra of a mixture of OMPD and 6-cyano-UMP confirmed the crystallographic finding of a covalent complex (Supporting Information, Figure S1–S3). Despite the current atomic resolution (1.1 Å) of the Asp312Asn OMPD–6-cyano-UMP complex it cannot be determined whether the structure represents an amidine **11** or an amide **12** (Scheme 1) due to a lack of electron density for polar hydrogen atoms around the amidine/amide group. Burial of a positively charged amidine is disfavored because the Asp312Asn mutant lacks a compensating negative charge. An amide would therefore result in a more stable OMPD complex, but in the absence of more data the amidine was modeled as it is the simpler adduct. The comparison of the reaction products from wt-OMPD and the Asp312Asn mutant raises the intriguing questions as to why a similar adduct was not obtained with wt-OMPD and where the water molecules that are required for hydrolysis come from. Once the amidine **11** is formed in wt-OMPD, it is converted to UMP within a matter of hours. Therefore, the active site in wt-OMPD must be accessible to water molecules from bulk solvent. The observation of a water molecule close to the C6-position in the OMPD–6-cyano-UMP product complex indicates that solvent might enter the OMPD active site during catalysis. Apparently the covalent complex in the Asp312Asn mutant is more stable than in wt-OMPD. If Asp312 acts as a general base for deprotonation of water to attack the amidine or amide bonds formed with Lys314, then the covalent intermediates were too unstable to be observed crystallographically in wt-

OMPD. By contrast, removal of the general base in the Asp312Asn mutant would result in a kinetic trap, enabling long-term population of the covalent intermediate.

After it was established that Lys314 acts as a nucleophile and water can access the OMPD active site during degradation of 6-cyano-UMP, the next question was what effects a Lys314Ala mutation would have on this reaction? Elimination of the nucleophile might be expected to result in an authentic and stable OMPD–6-cyano-UMP complex. Instead, the UMP complex was observed, similar to the result with wt-OMPD (Figure 2). Residual decarboxylase activity in a *M. thermoautotrophicum* OMPD mutant lacking the lysine has been reported.[13] The human Lys314Ala OMPD mutant also displays residual decarboxylase activity: a 1.1 Å resolution structure, which was obtained from cocrystallization of OMP with the K314A mutant, shows UMP only (data not shown because the outcome is the same as in the right panel of Figure 2). Apparently the functionality of the lysine residue, regardless of its involvement in decarboxylation or hydrolysis reactions, can be replaced by other parts of the enzyme or water. The residual activity of the Lys314Ala mutant is sufficient to slowly convert 6-cyano-UMP to UMP, but must involve a different mechanism than wt-OMPD. The likely course of action is mediated by water. Removal of the lysine side-chain leaves a cavity that is filled with water molecules that establish a hydrogen-bond network (Figure 2). Again, Asp312 might act as general base that activates water to convert the nitrile to the carboxylic acid. Another candidate for a general base is Asp317 (Figure 1). In summary, Lys314 is not absolutely required for decarboxylation and hydrolysis reactions of human OMPD. However, compared to water Lys314 is much more efficient in catalyzing these reactions.

Differential reactivity of 6-azido-UMP with OMPD mutants: To exclude singular observations that are peculiar to 6-cyano-UMP, an additional set of crystal structures with another nucleotide was essential. The azido and cyano groups are roughly isosteric, and both contain an electrophilic center. 6-Azido-UMP (**7**) is a covalent (suicide) inhibitor of *Plasmodium falciparum* OMPD.[7] 6-Azido-UMP was therefore used to address the question whether substrate distortion and reactivities similar to 6-cyano-UMP are also observed with this nucleotide, and whether these reactivities are dependent on the catalytic Lys314. Cocrystal structures of 6-azido-UMP were determined with wt-OMPD and the Asp312Asn and Lys314Ala mutants (Figure 3). The cocrystal structure of 6-azido-UMP and Lys314Ala OMPD revealed BMP as the product. Similar to the case of 6-cyano-UMP (see above), removal of the nucleophilic lysine is insufficient to arrest chemistry but alters the reaction pathway. A hydroxyl group is substituted for the azide moiety, and a water molecule that is present at the position of the former Lys314 could act as a nucleophile. Comparison of this structure with the wt-OMPD–BMP complex (Figure 3) revealed little conformational differences. As discussed for 6-cyano-UMP, in the absence of Lys314 water located close to this

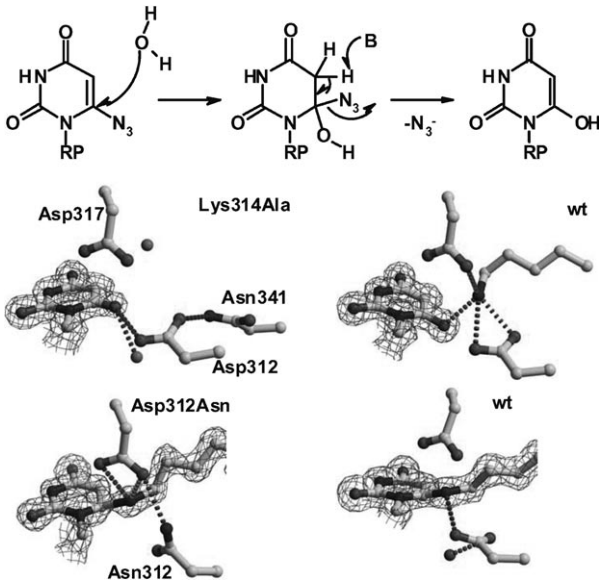


Figure 3. The OMPD mutant Lys314Ala converts 6-azido-UMP to BMP (middle left). A possible reaction scheme for this transformation is given at the top. The structure of wt-OMPD in complex with BMP (middle right) is similar to the Lys314Ala-BMP complex. A covalent adduct of UMP and Lys314 is seen in the Asp312Asn–6-azido-UMP complex (bottom left) and also in the wt-OMPD–6-azido-UMP complex (bottom right).

position adopts the role of the nucleophile with Asp312 and Asp317 as possible general bases. Both wt and Asp312Asn OMPD react with 6-azido-UMP (**7**) to form covalent bonds with the C6-position of UMP; this directly displaces azide (Figure 3). Taken together, the studies with 6-azido-UMP underscore the conclusions that were derived from 6-cyano-UMP. The azide moiety in 6-azido-UMP constitutes such a good leaving group that any nucleophile within the confinements of the OMPD protein will displace it. This result is in agreement with the analogous covalent inhibition of *M. thermoautotrophicum* OMPD by 6-azido-UMP.[7]

6-Acetyl-UMP reveals a higher nucleophilicity of Lys314 in Asp312Asn versus wt-OMPD: From the observed covalent complexes and water-induced reaction products with the 6-cyano-UMP and 6-azido-UMP nucleotides, it can be concluded that the OMPD active site has a propensity to allow for nucleophilic substitutions. The question arises as to which residues in the binding site might determine the nucleophilicity? To address this issue, 6-acetyl-uridine-5′-monophosphate (6-acetyl-UMP, **5**) was synthesized and reacted with wt-OMPD and the Asp312Asn variant. 6-Acetyl-UMP is isosteric with the substrate OMP, but cannot decompose to UMP. Similar to 6-cyano-UMP and 6-azido-UMP, the carbonyl group in 6-acetyl-UMP is electrophilic, but not prone to hydrolysis or substitution. This ligand is, therefore, suited to detect differences in nucleophilicity without being engaged in side-reactions such as hydrolysis.

The complex of wt-OMPD with 6-acetyl-UMP revealed an intact but distorted nucleotide, similar to the distortion

of OMP that was observed in the OMPD–Asp312Asn complex.[3] The acetyl group is tilted out of the plane of the pyrimidine ring, but no covalent interaction with Lys314 is present (Figure 4). The carbonyl group interacts through a hydrogen bond with the Lys314 side-chain. By contrast, the cocrystal structure of the Asp312Asn mutant and 6-acetyl-UMP clearly shows a covalent bond between the carbonyl group and the Nζ atom of Lys314, which forms an imine (Figure 4). The geometry of this adduct is very similar to the amidine that is formed with 6-cyano-UMP (Figure 2). The difference in reactivity of Lys314 in wt-OMPD and the Asp312Asn mutant stems from the removal of the negatively charged aspartate and the change in hydrogen-bonding capabilities by the introduced asparagine. In the wt structure, Asp312 forms hydrogen bonds to both Lys314 and Asn317. The direct contact with Lys314 leads to a charged hydrogen bond with the protonated amino group devoid of any nucleophilicity in the ground state. Upon mutation to asparagine, the hydrogen bond to Lys314 is retained but weakened due to a rotation of the Asn312 carboxamide group by 90°. The loss of a negative charge will reduce the pK_a and increase the nucleophilicity of Lys314, which will proceed to form a covalent complex.

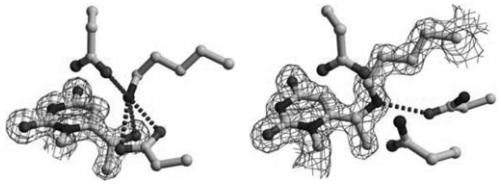


Figure 4. 6-Acetyl-UMP (**5**) in the active site of wt-OMPD (left) and covalently bound to *N*ζ of Lys314 of OMPD Asp312Asn (right).

Why is 6-cyano-UMP but not 6-acetyl-UMP a substrate for wt-OMPD? Molecular crowding of Lys314 with the C6 substituent would be expected to lead to a fast addition reaction that is mainly entropy-driven without significant substrate specificity. The OMPD–6-acetyl-UMP structure shows an antiparallel orientation of the ε-amino group of Lys314 and the carbonyl group in 6-acetyl-UMP. Nucleophilic attack requires approach of Lys314 along the Bürgi–Dunitz trajectory at an angle of 107°, which is impossible for the stereochemistry in the structure.[14,15] Such a limitation does not exist for a nitrile with rotational symmetry around the electrophile. The hydrogen bond of Lys314 to the carbonyl group of 6-acetyl-UMP is another possibility for reduced nucleophilicity, but there is no analogous interaction of Lys314 in the OMPD–6-cyano-UMP structure. Whereas the slow reaction of Lys314 with electrophiles will depend on molecular crowding in the OMPD active site, additional stereochemical and electrostatic requirements need to be fulfilled for a reaction to occur.

Regiospecificity and molecular determinants of substrate distortion: To investigate whether chemical transformations or substrate distortions by OMPD were limited to functionalities at the C6-position of the pyrimidine ring, the regioisomer 5-cyano-UMP (**6**) was chosen as a probe. 5-Cyano-UMP is a close homologue to 6-cyano-UMP and should experience similar forces upon binding to OMPD as 6-cyano-UMP. However, in the cocrystal structure with wt-OMPD, 5-cyano-UMP is intact and displays canonical geometry (Figure 5). In stark contrast to 6-cyano-UMP, no substrate distortion is present in 5-cyano-UMP, and the cyano group is coplanar with the pyrimidine ring. The nitrile displaces Lys314 by about 2.5 Å and fits well into a hydrophobic pocket that is lined by Ile368, Met371, and Ile401.[3] Thus, in contrast to the 6-position, the 5-position of pyrimidine nucleotides is not affected by ligand strain; this indicates that OMPD exerts ligand strain in a very localized fashion specifically onto the 6-position.[16]

Figure 5. Comparison of 5-cyano-UMP with 6-cyano-UMP. In case of the 5-cyano moiety no distortion of the substituent is present (left), whereas the cyano moiety in the wt-OMPD–6-cyano-UMP complex shows a strong distortion from planarity with the aromatic pyrimidine ring (right and see Figure 2).

The question arises whether the nature of the C6 substituent determines the extent of ligand strain. The C1−C6 torsion angle is a measure of the degree of tilting of the C6 substituent from the pyrimidine ring plane. A survey of the available human OMPD structures complexed to a C6-modified UMP derivative shows that this torsion angle seems to correlate with the van der Waals volume and structure of the C6 substituent. For instance, in the atomic resolution (1.24 Å) wt-OMPD–BMP structure, the C1-C6 torsion angle is only 2.4°, whereas this angle increases to 26.0° for the homologous 6-mercapto-UMP (**8**) (Figure 6 and Sup-

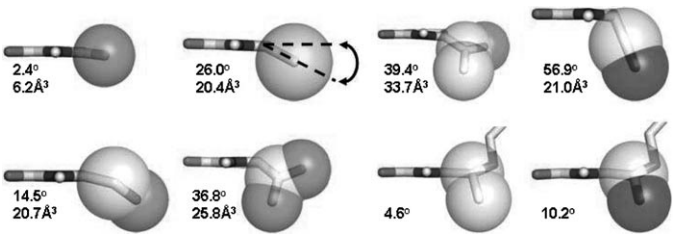


Figure 6. Distortion of substrates in wt (top) and Asp312Asn OMPD (bottom). Top from left to right: BMP, 6-mercapto-UMP, 6-acetyl-UMP, 6-cyano-UMP. Bottom: 6-Hydroxymethyl-UMP, OMP, 6-acetyl-UMP, 6-cyano-UMP. The view is along the glycosidic bond and the C1−C6 torsion angle is visible as the deviation from planarity as indicated by the dashed lines for 6-mercapto-UMP. The distortion in degrees and the difference in volumes of the undistorted ligands relative to UMP are given.

porting Information Figure S4). Despite comparable volumes, the 6-cyano group is bent twice as strongly (57°) as the mercapto group, possibly due to the longer "lever" and less polarizability of the nitrile compared to the thiol. The more flexible hydroxymethyl group, having the same volume as the cyano and mercapto group, is least distorted. Finally, comparison of the substituents acetyl and carboxyl that have similar shapes shows a comparable degree of distortion ($<40°$), indicating that charge does not influence the amount of ligand strain. In summary, it appears that ligand strain is maximal for large, non-polarizable, non-flexible substituents that extend far away from the pyrimidine ring. This information could prove useful for inhibitor design. By introducing a chiral center at C6 that carries a substituent that is prone to strong deformation in the context of a pyrimidine, the deformation energy would be gained as binding affinity.

Straining of small molecules by OMPD requires significant energy that might be stored either in the enzyme, or paid for by low binding affinities of the ligands. The K_i values of the nucleotides that do not form covalent complexes with wt-OMPD are all in the high μM range (Table 1). Ligand strain might be the reason for these low affinities, but UMP, which lacks a 6-substituent, also displays low affinity.[3] It thus appears that ligand distortion is energetically silent. A possible explanation is the engagement of new productive interactions after straining of the ligand. For instance, the kinked substituents of 6-mercapto-UMP and 6-cyano-UMP point into a hydrophobic pocket that is lined by OMPD residues Phe86, Ile177, and Ile 224. Being more strongly kinked, the nitrile fits much better into this pocket, and this might explain its increased affinity compared to the thiol. This notion is supported by the fact that the K_M value for OMP, which is strained when bound to OMPD, is 200-fold smaller than the K_i for UMP;[2a] these values indicate that the strained enzyme–substrate complex is more stable than the product complex and that the kinked carboxylate entertains favorable interactions. An additional explanation why unfavorable distortions of the substrate are tolerated is their overcompensation by strong energetic contributions from the ribose-phosphate part of the nucleotide (Circe effect).[17] It should be expected that the strain energy is released upon chemical transformation of the ligand, and this is indeed observed in the crystal structures. Upon covalent attachment of Lys314 in the Asp312Asn mutant to either the acetyl or cyano substituent, ligand strain is released. The torsion angles are only 5–10° and thus close to the situation of the stable BMP complex (Figure 6). For *M thermoautotrophicum* OMPD, electrostatic stress exerted by Asp70 (Asp312 in human OMPD) was suggested to prime OMP for decarboxylation.[3] Due to such electrostatic ground-state destabilization, OMPD is expected to exhibit affinity for nucleotides with positively charged C6 substituents. However, binding of 6-methylamino-UMP was found to occur in the neutral state, which would argue against electrostatic destabilization of the enzyme–substrate complex.[18] Although the exact mechanism of ligand strain and its relevance for OMPD catalysis is not known, there are now enough structural examples of OMPD complexes with strained ligands to assign ligand strain as a general property of OMPD. In this light, the removal of the kinked OMP carboxylate group might constitute an important driving force for decarboxylation.

Table 1. Approximate IC_{50} values for inhibitors of human OMPD at 25 °C.

Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)
6-mercapto-UMP	1680 ± 540	6-iodo-UMP[b]	25 ± 5
6-acetyl-UMP	24 ± 13	6-azido-UMP[b]	36 ± 17
6-cyano-UMP[a]	ca. 200	6-hydroxymethyl-UMP[c]	–
5-cyano-UMP	79 ± 36		

[a] Inhibition does not follow a simple Michaelis–Menten model. The IC_{50} value was estimated as the inhibitor concentration leading to a half-maximal reaction rate. [b] Pseudo-IC_{50} values because 6-iodo-UMP and 6-azido-UMP are covalent inhibitors. [c] No complete inhibition was achieved.

Whereas substrate distortion is a genuine property of OMPD, it is not limited to this enzyme. For instance, β-glycosidases deform their substrates to achieve a pseudoaxial orientation of the scissile bond.[19] In the crystal structure of the *B. subtilis* pyrophosphatase the pyrophosphate analogue PNP is deformed and the P–N bond is extended.[20] Finally, FTIR spectroscopy revealed substantial strain of the lactam carbonyl group in β-lactamase enzyme–substrate complexes.[21] A common feature of these examples is a saturated or more flexible substrate compared to the rigid pyrimidine nucleotide of OMPD. Distorting an aromatic ligand as seen in most OMPD crystal structures that are described here is expected to require considerably more energy than distorting a saturated or more flexible ligand, and it will be interesting to address the molecular mechanism leading to such unusual distortions.

Conclusions

Recent studies on OMPD from human, *P. falciparum*, and *M. thermoautotrophicum* have found that this enzyme in general can display rather peculiar chemistry that does not appear to bear any relation to the decarboxylation reaction.[3,8] These addition, substitution, and hydrolysis reactions are orders of magnitude slower than the native decarboxylation reaction, yet efficient enough to be readily observed. The chemodiversity is species-dependent because, for instance, 6-cyano-UMP degradation results in different products in human (see above) and *M. thermoautotrophicum* OMPD.[8] Likewise, the non-natural activities might be exploited for drug development, with the suicide inhibition of the malaria parasite *P. falciparum* OMPD by 6-iodo-UMP as an example.[3,4] All of these off-pathway activities rely on a key lysine residue, the functionality of which can only in part be replaced by water. The existence of such activities raises the question as to why OMPD harbors a nucleophile

that is apparently not used during catalysis but solely serves as a proton donor/acceptor? Recent carbon and deuterium isotope effect studies have provided evidence for a short-lived carbanion at the C6-position of the pyrimidine ring, which upon protonation, presumably by the lysine, gives the product UMP.[22] Apparently, the nucleophilicity of the lysine is harnessed in the ground state of the wild-type enzyme, but upon mutation of a nearby aspartic acid to asparagine, the nucleophilicity is increased as exemplified by the covalent linkage to the electrophilic groups of 6-cyano-UMP and 6-acetyl-UMP. This effect emphasizes the importance of the intact hydrogen-bonding network between charged side-chains in the OMPD active site for decarboxylation activity.[31]

Experimental Section

Protein purification, inhibitor studies, crystallization, and soaking experiments: OMPD was cloned, produced, purified, and crystallized as described.[23] Enzymatic activity, inhibitor studies, and cocrystallization experiments were performed as described in reference [5a]. Soaking experiments were performed for wt-OMPD with BMP and 6-cyano-UMP; and for Asp312Asn OMPD with 6-azido-UMP, 6-cyano-UMP, and 6-acetyl-UMP. The final nucleotide concentration in the crystallization drop was 2 mM, and soaking times varied from minutes (6-cyano-UMP) to several days (all other nucleotides).

Data collection, structure determination, and refinement: Data were collected at 100 K and reduced with the HKL programs (HKL research) or XDS.[24] The structures were determined by molecular replacement by using COMO and the best solution for the protein-only coordinates of previously published OMPD structures.[25,5a] Models were built in COOT and refined with REFMAC5.[26,27] The data that were derived from the 60 min soak of a wt-OMPD crystal with 6-cyano-UMP is slightly twinned with a twin operator ($l,-k,h$) and a twin fraction of 13%. Twinning in OMPD was described earlier,[23] and the wt-OMPD–6-cyano-UMP structure was refined against intensities by using a recent version of REFMAC5 that takes twinning into account. All other structures were derived from untwinned data. The same set of 5% of reflections was reserved for *R*-free cross-validation in all structures of a given crystal form.[28] Statistics are summarized in Table 1. Figures were created with Bobscript and rendered with Raster3D,[29,30] or PyMol (www.pymol.org). Volume calculations were done with Moloc (www.moloc.ch).

A detailed description of chemical syntheses, kinetics, and mass spectrometric data is provided in the Supporting Information.

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