Crystal Structure of Inhibitor Complexes Reveal an Alternate Binding Mode in Orotidine-5'-monophosphate Decarboxylase*

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The crystal structures of the enzyme orotidine-5'-monophosphate decarboxylase (ODCase)1 catalyzes the last step in the de novo pyrimidine biosynthesis pathway, converting OMP to UMP (Scheme I), which in turn serves as the source of all cellular pyrimidine nucleotides. ODCase continues to elicit strong interest not only because of its obvious importance in DNA and RNA synthesis, and thus in cell growth and proliferation, but even more so because of the enormous elusive nature of its reaction mechanism.

The decarboxylation of orotic acid in water of neutral pH is a very slow process (τ1/2 ~ 78 million years at 25 °C). The enzyme ODCase, however, catalyzes the breaking of this stable carbon-carbon bond at rates that are only 2 orders of magnitude below the limits set by diffusion. This astonishing feat qualifies ODCase as the most proficient enzyme known (1). Such catalytic power is even more remarkable if one considers that it is achieved without the help of any metal ions or cofactors and does not involve acid/base catalysis (2, 3). Not too surprisingly, several laboratories have made the chemical mechanism of this enzyme an object of their studies (4–8). Two strong inhibitors, 6-hydroxypyrimidine 5'-phosphate (barbituric acid ribosyl monophosphate; BMP) and 6-aza-UMP, are thought of as close mimics of the postulated carbanion intermediate thereby functioning as transition state analogues. Several weaker inhibitors include the product UMP and the purine nucleotides XMP and CMP (Scheme II). All of these compounds show a competitive inhibition pattern.

Recently published crystallographic studies of ODCases from four different organisms (9–12) identified the close conservation of their three-dimensional structures but still did not lead to a generally accepted mechanistic proposal (13–15). A Lys42-Asp70-Lys72-Asp708 arrangement creates a chain of alternating charges, which lies on one side of the binding pocket for the aromatic base. This cavity fits tightly around the bound product or inhibitor ligands (9–12). The substrate OMP itself, however, only enters this site in crystals of the double mutant D70A/K72A. Without the removal of the two side chains, there was insufficient space for substrate molecules to bind, with Asp70 not only overlapping in space but also exerting major electrostatic repulsion. In addition, there were no large conformational changes observed by either main chain or side chains when the wild-type enzyme and several active site mutants were crystallized in complex with substrate, product, and inhibitors (16). In light of this peculiar nature of the substrate-binding site, we have put forward the idea that stress created by charge-charge repulsion in a small rigid and hydrophobic environment was a major factor in rate acceleration. This stress would be expected to build up as the C6-carboxylate approaches the side chain of Asp70 leading to the release of CO2. The resulting carbanion would then be neutralized through proton transfer from the side chain of Lys72. The substantial binding energy generated by interactions of the phosphate and ribose parts of OMP with the enzyme matrix could provide a large part of the driving force for this process (9).

This model, however, does not explain why the purine nucleotide XMP with its bulkier two-ring base is a much better inhibitor of ODCase (K_i = 4.1 × 10^{-7} M) than the product UMP (K_i = 2.0 × 10^{-9} M), which in its chemical structure more closely resembles the strong inhibitor 6-aza-UMP (K_i = 6.4 × 10^{-9} M) (4). Given the remarkably tight fit to the binding site found in all structurally characterized pyrimidine nucleotide complexes (9–12, 16), one would have to postulate a major conformational change of the protein to accommodate the larger ring of XMP. The potential for such a reconfiguration of the active site would clearly weaken the argument for the involvement of electrostatic repulsion in the catalytic mechanism of the enzyme. We, therefore, set out to determine the
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binding mode of several well characterized inhibitor molecules, including XMP, to ODCase from the methanogenic archaeon *Methanobacterium thermoautotrophicum*.

In addition, we investigated the role played by the phosphate-binding loop, amino acids Gly
\(^{180}\)-Glu
\(^{190}\) in ligand binding by analyzing the 6-aza-UMP complex of the deletion mutant ∆R203A, in which amino acids 184–187 were removed and the phosphate anchoring Arg
\(^{203}\) was changed to alanine. The crystal structures of the complexes of wild-type ODCase with the product UMP and the most tightly binding inhibitor 6-hydroxyuridine 5′-phosphate (BMP) were also determined to allow meaningful comparisons with the other complexes studied by us but also with the results described by other groups for the enzymes from yeast (10), *Bacillus subtilis* (11), and *Escherichia coli* (12).

**EXPERIMENTAL PROCEDURES**

*Cloning, Protein Expression, and Purification—*Wild-type and mutant *M. thermoautotrophicum* ODases were expressed and purified as recently described (17). The ∆R203A mutant was constructed by first introducing a single site mutation, R203A (QuikChange™, Stratagene), by using the removal of the four residues Ala
\(^{184}\}-Gln
\(^{185}\)-Gly
\(^{186}\)-Gly
\(^{187}\) in a second round of mutagenesis. The coding strand sequences of the re-
the narrowness of the site that was to accept the aromatic rings of these molecules.

Its limited size, together with its resistance to conformational change, prevents the base-binding cavity from accepting the xanthine group of XMP (Fig. 1B). Unlike 6-aza-UMP, UMP, BMP, and OMP, XMP adopts the lower energy structure also found in solution, with $^{3/2}\text{H11032}$-endo sugar puckering and anti conformation of the base. Despite this change, Ser127 still serves as an anchoring residue for the nucleotide base forming two hydrogen bonds to xanthine. The side chain hydroxyl accepts a proton from N-1 and the backbone amide donates a proton to O-2. When compared with the complex of 6-aza-UMP and wild-type enzyme, the interactions between the phosphate group and Arg$^{203}$ as well as those of the 3'-OH of ribose with Asp$^{20}$ and Lys$^{42}$ were conserved. However, the 2'-OH group was no longer in contact with its usual partners, Asp$^{75B}$ and Thr$^{79B}$ from the other monomer. Instead, it was now only 3.0 Å away from the amino group of Lys42 and forms an additional bond to the C3-OH of (-)-1,3-butanediol, a crystallization additive which, together with a water molecule W1, is bound in the active site of the XMP-ODCase complex (Fig. 2A).

The molecule of butyl alcohol could be described to act like an adapter. Its hydrophobic chain fits nicely against the hydrophobic pocket of the active site, whereas its C1-OH binds to N-3 of xanthine, in addition to its C3-OH and the ribose 2'-OH interactions mentioned above. A superposition of the active sites of the XMP and 6-aza-UMP complexes (Fig. 3A) shows that water W1 assumes the regular binding spot of the ribose.

### Table I

|             | UMP  | BMP  | XMP  | CMP  | ΔR203A/6-aza-UMP |
|-------------|------|------|------|------|-----------------|
| Diffraction data |      |      |      |      |                 |
| Resolution (Å) | 1.5(1.55–1.5) | 1.6(1.63–1.6) | 1.9(1.97–1.9) | 1.9(1.97–1.9) | 1.9(1.97–1.9) |
| Measured reflections (n) | 425,800 | 355,820 | 376,089 | 765,425 | 489,020 |
| Unique reflections (n) | 34,387 | 28,942 | 32,685 | 32,661 | 58,208 |
| Completeness (%) | 95.2(73.9) | 97.0(92.8) | 99.0(99.9) | 99.9(99.9) | 90.6(64.0) |
| R cryst (%) | 3.0(16.7) | 3.0(8.5) | 8.5(31.2) | 4.0(10.0) | 4.8(28.3) |
| Space group | C222₁ | C222₁ | P2₁ | P2₁ | P1 |
| Molecules in asymmetric unit (n) | 1 | 1 | 2 | 2 | 4 |
| Refinement statistics |      |      |      |      |                 |
| Resolution (Å) | 30–1.5(1.59–1.5) | 30–1.6(1.7–1.6) | 30–1.9(2.02–1.9) | 30–1.9(2.02–1.9) | 30–1.9(2.02–1.9) |
| Protein atoms (n) | 1612 | 1594 | 3192 | 3196 | 6304 |
| Water molecules (n) | 193 | 191 | 180 | 159 | 206 |
| Reflections used for R cryst (n) | 2538(235) | 2309(372) | 1583(279) | 1326(216) | 1792(191) |
| R free (%) | 17.3(19.2) | 17.3(16.1) | 19.3(23.4) | 19.3(21.5) | 20.5(30.5) |
| Root mean square deviation bond length (Å) | 0.010 | 0.010 | 0.009 | 0.010 | 0.009 |
| Root mean square deviation bond angle (°) | 1.6 | 1.5 | 1.3 | 1.4 | 1.3 |
| Average B-factor (Å²) | 16.6 | 15.0 | 24.2 | 27.9 | 38.7 |
2′-OH, forming hydrogen bonds to OG1 of Thr^{79} and to the carboxylate of Asp^{75}.

The refined B-factor values for XMP (average B = 20.8 Å^2) indicate a high occupancy rate and low mobility of this molecule in the crystalline complex. For comparison, the average B-factor for the very rigid active site residues is 16.2 Å^2. Water W1 and the butyl alcohol molecule show significantly higher values of 34.9 and 43.6 Å^2, respectively, most probably indicative of increased mobility. It is interesting to note that both hydroxyls of the additive as well as the water W1 take up positions that are occupied by first-hydration shell water molecules in the free-enzyme structure.

**Kinetic Studies**—We performed kinetic measurements to establish whether the presence of (±)-1,3-butanediol had any major influence on catalysis. Increasing concentrations of alcohol do lower the apparent dissociation constant K_m from that measured with wild-type enzyme; V_max, however, stays constant (Table II). As the dielectric constant (ε) of the medium was decreased, ODCase binds the substrate more tightly. Ionic interactions and hydrogen bonds increase in strength with decreasing ε; therefore our results corroborate the idea that a large part of the substrate binding energy was generated from the interactions of the enzyme with the phosphate and the ribose groups of the nucleotide. Lowering ε will also make it easier to protonate the C6-carboxylate of OMP, minimizing potential electrostatic repulsion. Our results align well with the general finding that decarboxylation reactions are accelerated in desolvating environments (8).

At a fixed concentration of 200 μM XMP, increasing amounts of (±)-1,3-butanediol (5–20%, v/v) do not significantly change the values of V_max and K_m (Table II) leading us to believe that the alcohol does not drastically change the binding mode of XMP. The high B-factor values of the alcohol molecules also imply that their interactions with amino acids and the xanthine ring were not very specific and not very strong. Another argument for the general relevance of the orientation of the aromatic ring of XMP observed in crystals was the fact that the base of bound CMP adopts an almost identical position in the absence of any mitigating molecules (see below).

**Pyrimidine Inhibitor CMP**—The potential substrate 6-carboxy-CMP binds only very weakly to ODCase and its decarboxylation was almost undetectable (25); yet, there was no strong
structural reason for the absence of catalysis. When comparing
the interactions possible between the enzyme and orotate on
the one hand and a cytosine ring on the other, only one hydro-
gen bond might be lost in the latter (9). Most of the hydrogen
bonds formed (a water molecule linking the backbone carbonyl
group of Gln125 with O-4 of orotate and the hydrogen bond of
the latter to the backbone amide of Ser 127) could have the
partners act equally well as hydrogen donors or acceptors. In
complexes involving orotidine or uracil, the side chain hydroxyl
of Ser127 receives a bond from N3H of the base, and at the same
time, donates one to the oxygen of the amide side chain of
Gln185. However, it could only act as a donor to either one of
these positions in a potential CMP complex. To shed some light
on the structural basis of the rejection of CMP-carboxylate as a
substrate, we determined the crystal structure of the CMP
bound to

\[ \text{M. thermoautotrophicum} \]

ODCase. The crystal structure of the CMP complex reveals that the
pyrimidine nucleotide not only adopts a position very similar to
the one found for XMP but also engages in most of the inter-
actions displayed by the purine nucleotide. Another feature

\begin{table}[h]
\centering
\begin{tabular}{ l l l l l }
\hline
\textbf{(±)-1,3-Butanediol} & \textbf{\(V_{\text{max}}\)} & \textbf{S.E.} & \textbf{\(K_{m}\)} & \textbf{S.E.} \\
\hline
\textbf{A} & & & & \\
0 & 0.1968 & 0.0087 & 15.1704 & 2.2952 \\
5 & 0.1954 & 0.0077 & 14.9370 & 2.0245 \\
10 & 0.1933 & 0.0121 & 12.8725 & 2.9292 \\
15 & 0.1940 & 0.0114 & 10.5144 & 2.9292 \\
20 & 0.1925 & 0.0069 & 9.2132 & 1.3802 \\
\textbf{B} & & & & \\
0 & 0.1817 & 0.0063 & 26.7790 & 2.6082 \\
10 & 0.1866 & 0.0092 & 22.6105 & 3.3332 \\
12 & 0.1879 & 0.0066 & 22.6144 & 2.3628 \\
15 & 0.1932 & 0.0078 & 21.9065 & 2.6757 \\
20 & 0.1949 & 0.0078 & 24.0098 & 2.7837 \\
\hline
\end{tabular}
\caption{Kinetic measurements \textit{Effect of (±)-1,3-butanediol on catalytic activity of wild-type ODCase (A) and the effect of (±)-1,3-butanediol on XMP inhibition of wild-type ODCase (XMP) fixed at 200 \textmu M} (B).}
\end{table}
common to both complexes was the disordered state of the loop comprising residues Gly^{180}–Gly^{190}, the phosphate-binding loop (Fig. 1C).

Both active sites of the functional dimer were occupied by CMP molecules as well as by waters of increased mobility (Fig. 2B). CMP assumes its solution conformation, with the ribose ring in the 3′-OH endo position and cytosine oriented anti to the sugar ring (Fig. 3D), again mirroring XMP in its complex with ODCase. Both CMP molecules have relatively high B-factor values, 34.2 and 42.3 Å² for the A and B monomers of the homodimer, respectively, in contrast to 19.4 and 23.3 Å² for the conserved enzyme residues surrounding them. The binding modes of the two cytosine bases were not completely identical; the one in the A monomer assumes a position slightly closer to that of the 6-azauracyl ring in the 6-aza-UMP complex than does its counterpart in the B monomer. The increased mobility of CMP was accompanied by the disordering of the guanidinium head group of Arg^{203} in the active site of monomer A. While the ribose 3′-OH was still fixed by hydrogen bonds to Asp^{20} (2.7 Å) and Lys^{42} (2.8 Å), the 2′-OH group was not making its proper contacts with the enzyme but formed a long hydrogen bond with the amino group of Lys^{42} (3.1 Å) instead.

For the crystallization of the CMP-ODCase complex, 1,2,3-heptanetriol was used as an additive. As this 7-carbon alcohol is much larger than (+)-1,3-butane-diol, it cannot fit between the nucleotide and the active site wall. Instead, five water molecules fill the empty cavity designed to hold the base in the productive nucleotide-binding mode. One of them sits in the position that the 2′-OH would occupy in such a complex. It is equivalent to water W1 in the XMP complex and engages in the same interactions to Asp^{75B} and Thr^{79B}.

Impaired Phosphate Binding Mutant, ΔR203A—Kinetic studies have made it quite clear that the binding energy contributed by the 5′-phosphate group is very important for ODCase catalysis. Mutants with impaired phosphate binding ability have drastically reduced catalytic rates (4). To investigate the structural basis of this property, especially to find out whether the loss of the majority of bonds between the phosphate group and the enzyme has any effects on the conformation of the residues surrounding the aromatic rings of the nucleotide bases, we mutated Arg^{203} in the main phosphate-interacting residue, to alanine and deleted the four residues A^184QGGG^{187} of the phosphate-binding loop. Following co-crystallization, the structure of this mutant protein was determined in its complex with the inhibitor 6-aza-UMP. The reduced ligand binding affinity correlates well with a loss in crystal stability but does not lead to any structural adaptation of the active site. First, crystals of the complex form appear but over time many of them dissolve and ligand-free crystals (space group P4_1212) start to grow on their surfaces. The residual complex crystals start to bend. If complex crystals were harvested after nucleation of free enzyme crystals had started the resolution of their diffraction pattern dropped to about 5 Å. Both effects are consistent with the build up of stress in the crystal lattice. Given these observations, it came as no surprise to find that the refined overall B-factor value for this mutant complex is quite high (38.7 Å² averaged over the four monomers located in the asymmetric unit) with one of the monomers displaying an overall B-factor value of 44.6 Å². The electron density corresponding to the phosphate ligand in the deletion mutant is distinctly weaker and of lower quality than the density found for the XMP or CMP ligands in their respective complexes. Together with the problems encountered during the crystallization of the 6-aza-UMP-ΔR203A complex, this indicates an elevated tendency for ligand loss.

Although MgCl₂ had been added to the crystallization solution for stabilization, no defined metal ion was observed in the resulting 1.9-Å electron density map. Superposition of active site residues in the wild-type enzyme with their counterparts in the ΔR203A mutant (Fig. 3C) does not reveal any significant differences (root mean square deviations of 36 main chain and 9 side chain atoms = 0.12 Å). Despite its reduced affinity for inhibitors, the ΔR203A mutant binds 6-aza-UMP in exactly the same position as the wild-type enzyme (Fig. 1D). All interactions between the nucleoside part of the inhibitor molecule and active site residues were maintained, including the hydrogen bond between N-6 and Lys^{72} (3.1–3.2 Å). This was obviously not the case for the phosphate group. In addition to missing the ionic interaction with Arg^{203}, it has lost one of its first hydration shell waters that was held in place by the backbone amide of Gln^{185} and the backbone carbonyl of Val^{142} in wild-type ODCase.

One distinct difference between the structures of wild-type and deletion mutant enzymes was the presence of an additional, elongated electron density peak next to the azauracil ring in the latter (Fig. 2C). Whereas the average values of the B-factors for the active site residues and 6-aza-UMP range from 25 to 36 Å², the B-factor for the water molecule modeled into the density has a value of 60 Å². It was not clear whether this density represents alternate but overlapping binding positions of water molecules or whether it was caused by the low occupancy presence of an as yet unidentified low molecular weight compound.

BMP, 6-Aza-UMP, and UMP Inhibitor Complexes—BMP, 6-aza-UMP, and UMP all have almost identical molecular structures except for positions 6 of the respective bases. Both BMP (Kᵢ = 8.8 × 10⁻¹² M) (26) and 6-aza-UMP (Kᵢ = 6.4 × 10⁻⁸ M) (4) are strong inhibitors. This has been attributed to their similarity to the carbanion, which was postulated as a reaction intermediate stabilized by the positive charge of Lys^{72} (26). This single favorable interaction alone, however, cannot adequately explain why the product UMP is so ineffective as an inhibitor (Kᵢ = 2.0 × 10⁻⁴ M). The crystal structures of the three compounds complexed with various homologous ODCases were known (9–12). We decided to reanalyze the corresponding complexes with M. thermoautotrophicum ODCase as the crystals of this enzyme diffract to significantly higher resolution than do those from other sources. At the same time, the results of such analyses provide a common frame of reference against which the significant changes between the various ligand complexes can be assessed.

When the structures of the different M. thermoautotrophicum complexes were compared, only minimal changes were identified. The overall root mean square deviation value for 418 Ca atoms belonging to a dimer was less than 0.5 Å; the corresponding value was 0.2 Å for 50 backbone and Cβ atoms of the following conserved active site residues: Asp^{20}, Lys^{42}, Asp^{70}, Lys^{72}, Ser^{127}, Gln^{185}, Arg^{203}, Asp^{75B}, Ile^{76B}, and Thr^{79B}. The similarities even extend to water molecules accompanying the inhibitors in the binding site. As described for the structures of the enzymes from E. coli (12) and yeast (10), the negatively charged atom O-6 of BMP interacts electrostatically with Lys^{72} (2.8 Å) and at the same time via hydrogen bonding with a solvating water molecule and with OD1 of Asp^{70}.

As part of the uracil ring of UMP, C-6 does not carry a negative charge and cannot act as a partner in ionic or hydrogen bond interactions. Consequently, the amino group of Lys^{72} was located more than 1 Å away from its positions in the 6-aza-UMP-ODCase and BMP-ODCase complexes. The rest of the conserved side chains shows no changes in position. In addition to the loss of an energetically very favorable interaction with the side chain of Lys^{72}, the UMP complex structure
reveals another reason for the inefficiency of UMP to act as an inhibitor of ODCases. In the ligand-free structure, there was one well ordered solvent molecule for each of the charged residues in the active site. Binding of UMP creates a cavity between the inhibitor molecule and the enzyme surface because of desolvation. As the exact energy involved in such a process depends on the microenvironment it was extremely difficult to estimate. However, it will undoubtedly impose appreciable entropic cost on the formation of the UMP-ODCase complex, which in turn will lead to an increase in the inhibition constant.

**DISCUSSION**

All of the complex structures reported here should help to improve our understanding of the highly specialized and finely tuned active site of ODCase. There was only one direct interaction (the interaction with Lys72) between the active site of the enzyme and UMP that generates binding energy and was lost when compared with the strong inhibitors BMP and 6-aza-UMP. The xanthine ring of XMP does not interact with Lys72 either; nevertheless, this nucleotide is a rather good inhibitor of ODCase. We identified two entropic factors that could mitigate the effects of this missing interaction. Binding of XMP is not accompanied by the adoption of one fixed conformation by the loop Gly180-Gly190, and it does not lead to the stripping of water molecules from the charged residues in the active site. In addition, XMP itself retains the low energy conformation it assumes in solution.

Whereas UMP was much less tightly bound than the analogues that carry a (full or partial) negative charge close to position C-6 of the base, its aromatic ring enters the base-binding cavity and adopts a position common to those charge-carrying analogues. Somewhat surprisingly, given its close similarity to UMP, the base of CMP assumes a position almost identical to that of its counterpart in XMP. As mentioned above, the removal of a hydrogen atom from N-3 of CMP makes it impossible for this atom to act as a hydrogen bond donor. This, in turn, would lead to the loss of one hydrogen bond emanating from the side chain hydroxyl of Ser127. Whether this missing interaction. Binding of XMP is not accompanied by the adoption of one fixed conformation by the loop Gly180-Gly190, and it does not lead to the stripping of water molecules from the charged residues in the active site. In addition, XMP itself retains the low energy conformation it assumes in solution.

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