Atomic Resolution Structure of the Orotidine 5′-Monophosphate Decarboxylase Product Complex Combined with Surface Plasmon Resonance Analysis

IMPLICATIONS FOR THE CATALYTIC MECHANISM

Masahiro Fujihashi, Kazuya Mito, Emil F. Pai, and Kunio Miki

From the Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan and The Campbell Family Cancer Research Institute, Ontario Cancer Institute/University Health Network and Departments of Biochemistry, Medical Biophysics, and Molecular Genetics, University of Toronto, Toronto, Ontario M5G 1L7, Canada

Orotidine 5′-monophosphate decarboxylase (ODCase) accelerates the decarboxylation of its substrate by 17 orders of magnitude. One argument brought forward against steric/electrostatic repulsion causing substrate distortion at the carboxylate substituent as part of the catalysis has been the weak binding affinity of the decarboxylated product (UMP). The crystal structure of the UMP complex of ODCase at atomic resolution (1.03 Å) shows steric competition between the product UMP and the side chain of a catalytic lysine residue. Surface plasmon resonance analysis indicates that UMP binds 5 orders of magnitude more tightly to a mutant in which the interfering side chain has been removed than to wild-type ODCase. These results explain the low affinity of UMP and counter a seemingly very strong argument against a contribution of substrate distortion to the catalytic reaction mechanism of ODCase.

Orotidine 5′-monophosphate decarboxylase (ODCase) is one of the most proficient enzymes known (1). It decarboxylates orotidine 5′-monophosphate (OMP) and produces uridine 5′-monophosphate (UMP) in the final step of the de novo pyrimidine biosynthesis pathway (Fig. 1A). It also accelerates the reaction by 17 orders of magnitude, as compared with the spontaneous reaction in water at neutral pH, without employing cofactors or metal ions (1–5).

The reaction mechanism of this enzyme has been the subject of extensive investigations. More than 170 crystal structures have been determined, and numerous kinetic assays at various conditions have been performed. These experiments established that an electrostatic residue network composed of the charged side chains of two aspartate and two lysine residues, all completely conserved, plays a dominant role in catalysis (Fig. 1B) (2–5). As with all enzymes, the reaction acceleration provided by ODCase is explained in part by transition state stabilization (6). Lys-72 (the sequence numbers in this study correspond to those of the ODCase from Methanothermobacter thermautotrophicus (MtODCase)) is considered to be the key residue to stabilize the intermediate vinyl anion (6, 7). However, there is still no general agreement on all the details of the reaction mechanism. In particular, the observation that ODCase also converts 6-cyano-UMP into 6-hydroxy-UMP at the same site where the decarboxylation reaction occurs (Fig. 1A) (8) complicates the scenario. The environment required to stabilize the vinyl anion does not seem suitable to support, at the same time, the intermediate of the side reaction because the release of a negatively charged cyano group would leave a positive intermediate, the opposite of a carbanion.

A possible contributor to the acceleration of a broader array of reactions may be substrate distortion (5, 9, 10). Such a distortion effect was observed in crystal structures of various ODCase-ligand complexes, such as OMP, 6-aceto-UMP, and 6-cyano-UMP, with both MtODCase and human ODCase (11–14). For 6-cyano-UMP, such a distortion receives strong support from Raman spectroscopy, which indicates bond bending of about 20° (15). The crystal structure of ODCase from Plasmodium falciparum in complex with OMP (16) implied that charge repulsion between the two carboxylates of Asp-70 and OMP is a prime candidate for causing such a distortion.
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EXPERIMENTAL PROCEDURES

The binding affinity of UMP, however, is significantly weaker than that of the substrate OMP and other UMP derivatives with negatively charged substituents at C6 (17–20), an obviously serious argument against such an interpretation (18, 21). The low affinity of UMP seems inconsistent with the substrate distortion mechanism because UMP has lost its carboxylate group and with it the main candidate for repulsion with Asp-70. To investigate this inconsistency, we determined the crystal structure of the complex of MtODCase and UMP at atomic resolution, hypothesizing that the reason why UMP does not strongly bind to ODCase should be reflected in the molecular structure. We also undertook surface plasmon resonance analyses of wild-type ODCase and both the K72A and the K42A mutants of ODCase to further probe the roles of residues that played in catalysis in the context of the atomic resolution structure. The results of the combined investigations revealed a repulsive interaction between the side chain of Lys-72 and UMP, explaining the low affinity of the reaction product and removing a strong argument against significant contributions of substrate distortion to the enormous rate acceleration observed in ODCase catalysis.

EXPERIMENTAL PROCEDURES

Materials—Uridine 5'-monophosphate disodium salt was purchased from Nacalai Tesque (Kyoto, Japan). 6-Azauridine 5'-monophosphate (6azaUMP) was purchased from Carbosynth (Compton, UK). HBS-N buffer and series S sensor chip for 420 s at a flow rate of 10 μl/min followed by an ethanolamine hydrochloride solution treatment. 2,000–4,000

Odyssey catalyzing reaction and its reaction center. A, substrates and products of decarboxylation and cyano-converting reactions. B, reaction center of MtODCase in complex with 6-hydroxy-UMP (BMP, Protein Data Bank (PDB): 1lor (29)). Transparent spheres indicate the van der Waals radii of the four charged side chains Lys-42, Asp-70, Lys-72, and Asp-75' (′ indicates that the residue belongs to the second subunit of the dimeric enzyme). Numbers beside the dotted lines represent the distances between the connected atoms in Angstroms.

Crystallography—Crystallization was performed at 293 K using the hanging-drop vapor diffusion method. A solution of buffer A containing 10 mg/ml WT-MtODCase and 10 mM uridine 5'-monophosphate (UMP) was mixed with equal amounts of precipitant solution composed of 1.28 M sodium citrate, 5% (v/v) dioxane, and 0.1 M HEPES-Na, pH 7.5. Crystals grew as clusters, and several cycles of microseeding were necessary to obtain a single crystal. Crystals were dipped in a cryoprotectant buffer consisting of 1.2 M sodium citrate, 15% glycerol, and 0.1 M MES-Na, pH 6.5, before being flash-frozen in a stream of nitrogen at ~100 K.

Various attempts to obtain an atomic resolution dataset of MtODCase with UMP were taken at beamlines of the Photon Factory and SPring-8, Japan, as well as at the Advanced Photon Source. The dataset eventually used in structure refinement was collected at beamline 14BM-C of the BioCARS sector at the Advanced Photon Source. The diffraction data were integrated and scaled using HKL2000 (23). The crystals displayed the same space group and unit cell constants as the previously determined structure of the K72A complex of 6-aminoUMP, which was phased by the molecular replacement method with the program MOLREP (39) using the WT in complex with 6azaUMP (1dvj, Ref. 5). Thus, the coordinates of K72A and 6-aminoUMP complex were used as the initial model for refinement. Small changes to the model were accomplished using the program COOT (24). The refinement was started using the program REFMAC (25), and the program SHELEX (26) was applied at the later stages. Anisotropic B factors were assigned for all non-hydrogen atoms including solvent molecules. The bonds and angles of the side chains of Lys-42, Asp-70, Asp-75, and the pyrimidine ring of the ligand were unrestrained at the final refinement stage. The restraint parameters for Lys-72 were not changed from the default values due to its assuming dual conformations. The occupancies of the conformations A and B of Lys-72 were refined in the program SHELEX at the final stage of the refinement, whereas those of other residues were estimated based on the corresponding electron densities. The refined structure was validated using MolProbity (27). Statistics are summarized in Table 1.

Surface Plasmon Resonance Analysis—Surface plasmon resonance assays were performed using a Biacore T100 (GE Healthcare). WT and mutant MtODCase proteins were immobilized on sensor chips (Series S sensor chip CM5) using the amine coupling method and following the standard protocol (28). The MtODCase solutions were diluted to 0.1 mg/ml in 10 mM acetate buffer, pH 5 and 4.75 for WT and mutants, respectively, prior to immobilization. The sensor chips were activated by treating them with a mixture of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide and N-hydroxsuccinimide. The protein solutions were then brought into contact with the sensor chip for 420 s at a flow rate of 10 μl/min followed by an ethanolamine hydrochloride solution treatment. 2,000–4,000 resonance units of MtODCases were immobilized on the sensor chips.
The binding assays were performed at 25 °C and at a flow rate of 30 μl/min using HBs-N buffer composed of 10 mM HEPES-Na, pH 7.4, and 150 mM NaCl. The measurements were performed in triplicate for each combination of WT/K42A/K72A and UMP as well as WT and 6azaUMP. The K_D values for WT and K42A mutant were calculated with the affinity analysis option using Biacore T100 evaluation software version 1.1.1. The K_D values for the K72A mutant were estimated with the kinetics evaluation option of the same program.

RESULTS AND DISCUSSION

The crystal structure of the ODCase-product complex was determined at an atomic resolution of 1.03 Å (the highest resolution of all ODCase structures determined today) and refined to R_cry (R_free, factors of 11.8 and 13.9%, respectively (Table 1). Double conformers were assigned in 57 out of 215 modeled residues. Anisotropic B values of all individual atoms were refined, and 688 out of a possible 1,658 hydrogen atoms were added to the model structure. The side chains of Lys-42, Asp-70, and Asp-75 and the pyrimidine ring of UMP were unrestrained to avoid bias in the analysis of atoms in the ligand-binding site. The root mean square distances between the present and previously determined WT-MtODCase-UMP complex structures (1LOQ (29) and 3G1D (9), both at 1.5 Å resolution) are 0.32 Å (all 209 Ca) and 0.43 Å (all 215 Ca), respectively.

Our high resolution structure reveals the binding mode of UMP and its interactions with the protein matrix in close detail. As shown in Fig. 2, the side chain of Lys-72 assumes two conformations. In the first conformation (occupancy 54%, green in Fig. 2), the Ce and Nε atoms of the residue are located 3.26 and 2.78 Å, respectively, from C6 of UMP, whereas in the second conformation (occupancy 46%, orange in Fig. 2), the corresponding distances are 3.20 and 3.90 Å. Both distances in context.

![Figure 3: Ligand binding site. A, stereo view of UMP and both conformations of Lys-72 superposed on the Fo-Fc, omit electron density map of UMP contoured at 12 σ. Conformations A and B of Lys-72 are drawn in green and orange, respectively. A red arrow points to the C6 atom of UMP, which is displaced from the plane of the pyrimidine ring by 0.10 Å. B, the Lys-42-Asp-70-Lys-72-Asp-75 network in the present atomic resolution structure. Numbers beside the dotted lines represent the distances between the two connected atoms in Angstroms. The color code is the same as in panel A.](image)

![Figure 2: Omit electron density maps of Lys-72 superposed on its double conformations. Hydrogen atoms are drawn in white. Numbers beside the blue dotted lines represent the distances between the two connected atoms in Angstroms. A, conformation A. The light blue mesh shows the F – Fc, omit electron density map of Lys-72 contoured at 5.0 σ. Conformations A and B of Lys-72 are drawn in green and transparent gray, respectively, B, both conformations A and B. The light blue mesh represents the F – Fc, omit electron density map of Lys-72 contoured at 4.5 σ. Conformations A and B of Lys-72 are drawn in green and orange, respectively. C, conformation B. The light blue mesh represents the F – Fc, omit electron density map of conformation B of Lys-72 contoured at 3.5 σ. Conformations A and B of Lys-72 are drawn in transparent gray and orange, respectively.](image)
formation A are shorter than the sum of the van der Waals radii, as is the contact involving C/H9280 in conformation B. In the second conformation, UMP even seems to push Lys-72 out of its original position. In addition, the pyrimidine ring structure of UMP is slightly distorted with the C6 atom located out of the plane of the pyrimidine ring by 0.10 Å, whereas the remaining five ring atoms deviate by less than 0.015 Å (Fig. 3A). The Lys-42-Asp-70-Lys-72-Asp-75/H11032 network shown in Fig. 1B is kept intact in both of the two conformations (Fig. 3B). The structural features imply that UMP and Lys-72 compete to take their favorite positions of lowest energy in the active site of the enzyme.

This finding encouraged us to investigate the binding affinity between the K72A mutant of ODCase and UMP. So far, no K_i for UMP had been reported for this mutant because of its extremely low enzymatic activity (30). To overcome this problem, we performed K_D assays using the surface plasmon resonance technique.

WT-MtODCase was immobilized on a Biacore sensor chip. UMP and 6azaUMP were applied individually to the sensor chip. These compounds resulted in the sensorgrams shown in Fig. 4, A and B, left panels; the corresponding K_D values were calculated with the affinity analysis option of the Biacore T100 evaluation software. They are displayed in Fig. 4, A and B, right panels. The estimated value for UMP is 4.2 ± 0.2 × 10^-2 M (Table 2), which is comparable with the published K_i for UMP of 3.3 ± 0.1 × 10^-2 M at 55 °C (20). The K_D value for 6azaUMP is 2.9 ± 0.2 × 10^-6 M, approximately one-fourth of the published K_i for this compound (1.24 ± 0.07 × 10^-5 M at 55 °C (20)) (Table 2). These numbers show that K_D analysis using Biacore is able to at least estimate the order of magnitude of K_i values.

Next, we determined the K_D of UMP for the MtODCase K42A and K72A mutants. The K_D of UMP for the K42A mutant is 8.1 M (Fig. 4C and Table 2), which is an order of magnitude larger than the one seen with the WT enzyme. This value is reasonable because removal of the Lys-42 side chain leads to loss of the interaction between enzyme and the 2'-OH of ribose (9, 29). In contrast, the K_D of UMP for the K72A mutant is 4.1 ±
catalytic features have been well established, ODCase a target of intense scrutiny. Several of the underlying transition state stabilization as explanations for the high reduction in catalytic potency of the K72A mutant enzyme. The most remarkable acceleration of the decarboxylation it catalyzes has made the detailed chemical mechanism of ODCase a target of intense scrutiny. Several of the underlying catalytic features have been well established, e.g. the strong contribution of the 5′-phosphate group (30, 37, 38) or the vinyl carbanion nature of a reaction intermediate (6, 7). It is also generally assumed that Lys-72, which is completely conserved in all known ODCase sequences (16), plays an important role in balancing the negative charge of the vinyl carbanion and thereby lowers the energy of the transition state. The amino head group of the residue closely approaches the C6 atom of the pyrimidine rings of ligands (2–5, 11, 29), and its mutation to an alanine decreases the decarboxylation rate by 5 orders of magnitude (30). We now identify a close interaction between UMP and Lys-72 in the active site of the enzyme as the structural basis for the low affinity of WT-ODCase for its product. This finding counters a seemingly strong argument brought forward against the participation of substrate distortion in ODCase catalysis. As several earlier studies also support such a contribution (5, 9, 14, 15), it is highly probable that ODCase uses both transition state stabilization and substrate distortion (plus potentially other still unknown mechanistic features), with all of them needed to achieve the extraordinary rate acceleration of the enzyme.

It is reasonable to assume that ODCase makes use of a number of catalytic effects to achieve its high proficiency in decarboxylation reaction. In contrast, with an active site not evolved to support the generation of cationic intermediates, the enzyme is restricted to the use of substrate distortion when catalyzing the cyano-converting reaction described earlier (8), resulting in a rather slow rate for this reaction. Further investigations, such as theoretical studies based on the present experimental results, promise to shed more light on the mechanistic features of substrate distortion and how exactly it might contribute to ODCase catalysis.

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