

# Dissecting a Charged Network at the Active Site of Orotidine-5'-phosphate Decarboxylase\*

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**The crystal structure of yeast orotidine-5'-phosphate decarboxylase in complex with the postulated transition state analog, 6-hydroxyuridine-5'-phosphate, reveals contacts between this inhibitor and a novel quartet of charged residues (Lys-59, Asp-91, Lys-93, and Asp-96) within the active site. The structure also suggests a possible interaction between O<sub>2</sub> of the 6-hydroxyuridine-5'-phosphate pyrimidine ring and Gln-215. Here we report the results of mutagenesis of each of the charged active site residues and Gln-215. The activities of the Q215A and wild-type enzymes were equal indicating that any interactions between this residue and the pyrimidine ring are dispensable for efficient decarboxylation. For the D91A and K93A mutant enzymes, activity was reduced by more than 5 orders of magnitude and substrate binding could not be detected by isothermal calorimetry. For the D96A mutant enzyme, *k*<sub>cat</sub> was reduced by more than 5 orders of magnitude, and isothermal calorimetry indicated an 11-fold decrease in the affinity of this enzyme for the substrate in the ground state. For the K59A enzyme, *k*<sub>cat</sub> was reduced by a factor of 130, and *K*<sub>m</sub> had increased by a factor of 900. These results indicate that the integrity of the network of charged residues is essential for transition state stabilization.**

Orotidine-5'-phosphate decarboxylase (ODCase,<sup>1</sup> EC 4.1.1.23) produces a large rate enhancement, accelerating the rate of spontaneous decarboxylation of orotic acid derivatives by more than 17 orders of magnitude (see Ref. 1 and Fig. 1). The remarkable catalytic power of this enzyme is entirely dependent on non-covalent binding forces and does not involve metals or other cofactors (2, 3). In an attempt to gain insight into its mechanism of action, the crystal structures of ODCase complexed with three different ligands were recently determined (4–7). In each of these structures, a unique arrangement of alternating, charged residues composed of Lys-Asp-Lys-Asp is positioned near the bound pyrimidine ring of the ligand. These four residues, Lys-59, Asp-91, Lys-93, and Asp-96 of the yeast enzyme (see Fig. 2), are completely conserved in all known ODCase sequences (8). In the present work, we sought to determine their roles in catalysis.

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<sup>1</sup> The abbreviations used are: ODCase, orotidine-5'-phosphate decarboxylase; BMP, 6-hydroxyuridine-5'-phosphate; OMP, orotidine 5'-phosphate; MOPS, 4-morpholinepropanesulfonic acid; ES, enzyme-substrate.

The spontaneous decarboxylation of orotidine-5'-phosphate is very slow indeed (*k*<sub>non</sub> = 2.8 × 10<sup>-16</sup> sec<sup>-1</sup>). According to the theory of absolute reaction rates, a large value of *k*<sub>cat</sub>/*K*<sub>m</sub> (for ODCase = 6.3 × 10<sup>7</sup> M<sup>-1</sup> sec<sup>-1</sup>) requires that the altered substrate be very tightly bound in the transition state *K*<sub>tx</sub> < 10<sup>-23</sup> M (1). Moreover, a large value of *k*<sub>cat</sub> (for ODCase = 44 s<sup>-1</sup>) requires that the substrate be much less tightly bound in the ground state (1), as was recently established (*K*<sub>m</sub> = *K*<sub>s</sub> = 7 × 10<sup>-7</sup> M) (11, 13). From a structural standpoint, it would be desirable to understand how these very different affinities are achieved.

Based on computer simulations, it has been proposed (5, 6) that Asp-91 (yeast numbering system) may assist decarboxylation by a mechanism involving electrostatic destabilization of the substrate in the ground state. However, the likelihood of that mechanism of destabilization has been questioned (10). Lys-93 has been shown to be important for enzyme activity (9), possibly by stabilizing the carbanion produced by CO<sub>2</sub> elimination and furnishing the proton that appears at C-6 of the product UMP (Fig. 1). The second acidic member of the charged quartet, Asp-96, is contributed by the opposite subunit of the ODCase dimer (4–7) and is drawn into the active site by ligand binding. The final member of the quartet, Lys-59, appears to contact the 3'-hydroxyl group of the bound substrate (5–7).

To assess the contribution of each of these four residues to ground state and transition state stabilization, we have replaced each one with alanine. We also substituted alanine for Gln-215, a residue that appears to be within hydrogen-bonding distance (2.6 Å) of the C-2 oxygen of the BMP pyrimidine ring, possibly aiding delocalization of negative charge in the transition state for decarboxylation (4). Here we show that removal of any member of the charged network drastically reduces activity. In contrast, mutagenesis of Gln-215 reveals that this residue contributes very little to transition state stabilization.

## EXPERIMENTAL PROCEDURES

Mutant recombinant *ura3* genes encoding K59A, D91A, K93A, D96A, and Q215A enzymes were generated by site-directed mutagenesis using Quick-Change reagents (Stratagene, Inc.) and mutagenic oligonucleotide pairs (Oligos, Etc.). Wild-type and mutant enzymes were expressed and purified from *Escherichia coli* SS6130 (*cytR*, *Δcdd*) as previously described (11). Analysis of each mutant protein, either intact or as protease-generated peptides, by electrospray mass spectrometry demonstrated the correct alanyl substitution at positions 59, 91, 93, 96, and 215. Enzymatic decarboxylation of OMP was measured in MOPS buffer (2.0 × 10<sup>-2</sup> M, pH 7.2) by observing the decrease in absorbance at either 285 or 295 nm where Δε<sub>M</sub> = -1743 and -819 cm<sup>-1</sup>, respectively. An alternative assay (12), monitoring the evolution of <sup>14</sup>CO<sub>2</sub> from radiolabeled OMP, was used to estimate values of *K*<sub>m</sub> for wild-type and Q215A enzymes. Concentrations of wild-type and mutant enzymes were estimated from absorbance readings at 280 nm, using a molar extinction coefficient of 28,830 cm<sup>-1</sup>. Ligand binding affinities were determined by comparing rates of decarboxylation in the presence and absence of varying concentrations of each competitive inhibitor. Isothermal titration calorimetry was performed on a Microcal, Inc. MSC calorimeter equilibrated at 25 °C with a final enzyme concentration of 10<sup>-4</sup> M.

Circular dichroism spectra were obtained at 25 °C using an Applied Photophysics  $\pi^*$  180 spectrophotometer following dilution of each enzyme into potassium phosphate buffer ( $10^{-2}$  M, pH 7.2).

#### RESULTS AND DISCUSSION

The crystal structure of yeast ODCase complexed with BMP suggested the possibility of a hydrogen bond between Gln-215 and O<sub>2</sub> of the pyrimidine ring (see Ref. 4 and Fig. 2). However, the data in Tables I and II show that replacement of Gln-215 with alanine scarcely affects either catalysis or ligand binding. This result is surprising considering the closeness of the Gln-215 side chain to O<sub>2</sub> of the pyrimidine ring and indicates that any interactions between this residue and the pyrimidine ring are dispensable for efficient decarboxylation. Aside from contacts with the 6-substituent, the only remaining contact between the enzyme and the pyrimidine ring is formed between O-4 of the pyrimidine ring and the peptide bond amide group of Ser-154 (4).

Table III compares the activity of wild-type yeast ODCase with the activities of the K59A, D91A, K93A, and D96A mutant enzymes. In these assays, which contained substrate at a concentration 350-fold greater than the  $K_m$  value for wild-type ODCase ( $2.5 \times 10^{-4}$  M), no detectable activity was observed for the D91A, K93A, or D96A mutant enzymes, even at an enzyme

concentration up to  $2.0 \times 10^{-4}$  M in subunits. These experiments indicated that the activity of each mutant enzyme had decreased by more than 5 orders of magnitude compared with the wild-type enzyme (Table III). The loss of activity resulting from mutagenesis of Asp-91, Lys-93, and Asp-96 does not appear to be a consequence of gross changes in enzyme structure, as indicated by comparison of the CD spectra of mutant and wild-type enzymes.

In view of the negligible levels of enzyme activity observed for these mutant enzymes, it was of interest to determine whether they were capable of binding OMP. For the D91A and K93A mutant proteins, OMP binding could not be detected by isothermal titration calorimetry. In the case of the D96A protein, isothermal titration calorimetry yielded a value of  $8.0 \times 10^{-6}$  M for the binding affinity of OMP (Table I). This value is similar to the  $K_m$  value of the wild-type enzyme for 2'-deoxyrotidine 5'-phosphate determined in a separate study.<sup>2</sup> Earlier work has shown that, for the wild-type enzyme,  $K_m$  represents the dissociation constant of the enzyme-substrate complex (11, 13). The ability of the D96A protein to bind ligand, in contrast to the results obtained for the D91A and K93A proteins, is of interest in view of the fact that Asp-96 is contributed to the active site by the opposite subunit of dimeric ODCase upon ligand binding. Based on the activity limit and substrate binding affinity of the D96A protein, the apparent  $k_{cat}/K_m$  value for this mutant enzyme was reduced by more than 6 orders of magnitude. The absence of detectable substrate binding by the D91A and K93A mutant enzymes precludes the assessment of the effects of these substitutions upon the stability of the ES complex. However, the lack of activity of the D91A, K93A, and D96A mutant enzymes indicates that each of these charged residues play a critical role in transition state stabilization.

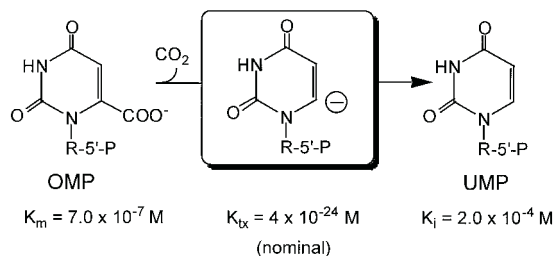


FIG. 1. The reaction catalyzed by orotidine-5'-phosphate decarboxylase.

<sup>2</sup> Miller, B. G., Butterfoss, G. L., Short, S. A., and Wolfenden, R. (2001) *Biochemistry*, in press.

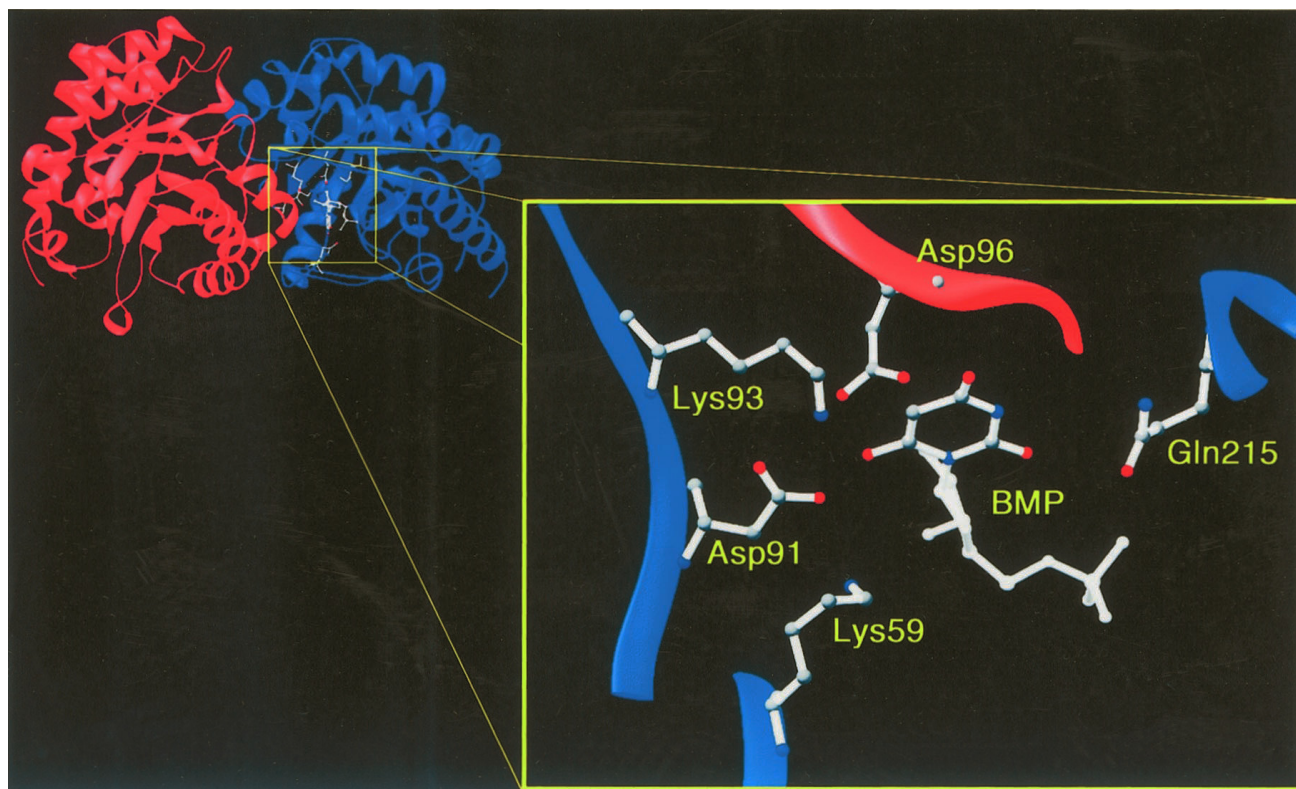


FIG. 2. Interactions between ODCase active site residues and the postulated transition state analog, BMP, as revealed by the crystal structure of the enzyme-inhibitor complex (4). The ODCase subunit containing Lys-59, Asp-91, and Lys-93 is in blue whereas the second subunit that contributes Asp-96 to the active site upon ligand binding is drawn in red. In this drawing, the ribose and phosphate moieties of BMP are shown in white. The dimer and active site close-up were rendered using Ribbons (16).

TABLE I  
Kinetic parameters of wild-type and mutant yeast ODCases

Enzyme	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_m$ $M$	$k_{\text{cat}}/K_m$ $M^{-1} \text{s}^{-1}$	$\Delta\Delta G$ $\text{kcal/mol}$
Wild-type	44	$7.0 \times 10^{-7}$	$6.3 \times 10^7$	0
Q215A	41	$2.5 \times 10^{-6}$	$1.6 \times 10^7$	0.81
K59A	0.34	$6.4 \times 10^{-4}$	$5.3 \times 10^2$	6.9
D96A	<0.00023	$8.0 \times 10^{-6a}$	<29	>8.6

<sup>a</sup> This value represents the dissociation constant of the OMP-D96A complex, determined by isothermal calorimetry.

TABLE II  
Ligand dissociation constants (mol/liter) for wild-type and mutant yeast ODCases

Enzyme	$K_i$ (UMP)	$K_m$ (OMP)	$K_i$ (XMP)	$K_i$ (6-AzaUMP)	$K_i$ (BMP)
Wild-type	$2.0 \times 10^{-4}$	$7.0 \times 10^{-7}$	$4.1 \times 10^{-7}$	$3.6 \times 10^{-8}$	$8.8 \times 10^{-12a}$
Q215A	$1.7 \times 10^{-4}$	$2.5 \times 10^{-6}$	$4.6 \times 10^{-7}$	$2.9 \times 10^{-8}$	ND <sup>b</sup>
K59A	$3.3 \times 10^{-4}$	$6.4 \times 10^{-4}$	$7.3 \times 10^{-4}$	$3.9 \times 10^{-5}$	$9.4 \times 10^{-8}$

<sup>a</sup> From Ref. 17.

<sup>b</sup> Not determined.

TABLE III  
Specific activities of purified wild-type and mutant yeast ODCase enzymes determined at a substrate concentration of  $2.5 \times 10^{-4} M$

Enzyme	Specific activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )
Wild-type	90
Q215A	84
K59A	0.19
D91A	$\leq 0.00066$
K93A	$\leq 0.00018$
D96A	$\leq 0.00047$

The crystal structures of all ODCase-ligand complexes suggest the presence of a hydrogen bond between the  $\epsilon$ -amino group of Lys-59 and the 3'-OH group of the ligand (4–7). Whereas removal of the side-chains of Asp-91, Lys-93, and Asp-96 completely destroyed activity, the enzyme retained measurable activity after mutagenesis of Lys-59. Table I shows that the  $k_{\text{cat}}$  value for the K59A mutant enzyme was reduced 100-fold whereas  $K_m$  was elevated 1000-fold compared with these parameters for the wild-type enzyme.

Table IV reveals a striking disparity between the magnitude of the effect of the Lys-59  $\rightarrow$  Ala substitution on the binding affinities of the substrate in the ground state and the altered substrate in the transition state. As the reaction progresses from the ground state ES complex to the transition state, the effect of the K59A mutation increases from 910-fold to  $1.2 \times 10^5$ -fold. Upon formation of product UMP, the magnitude of this effect collapses to less than 2-fold. These findings are consistent with the view that the active site of this enzyme is organized to maximize the influence of multiple binding interactions in the transition state, while minimizing such effects in the ground state and product complexes. The differential effect of the Lys-59  $\rightarrow$  Ala substitution upon ligand binding is comparable with, but somewhat exceeds, the effect of removing the 2'-OH group of the ligand, consistent with the view that interactions between enzyme and ribosyl OH groups are important for transition state stabilization.

To the extent that enzymatic decarboxylation depends on ground state destabilization, one would expect that any enzyme mutation that reduces this destabilization would tend to both reduce  $k_{\text{cat}}$  and increase the affinity of the enzyme for the substrate in the ground state. In fact, both the K59A and D96A mutant enzymes show reduced values of  $k_{\text{cat}}$  and reduced affinities for the substrate in the ground state. Similarly, both the D91A and K93A mutants were inactive, and isothermal

TABLE IV  
Relative contribution of Lys-59-ribofuranosyl interactions to substrate, inhibitor, and transition state binding affinity

Ligand	$K_i$ (K59A)/ $K_i$ (wild-type)
UMP	1.7
OMP	910 <sup>a</sup>
6-AzaUMP	1100
XMP	1800
BMP	11,000
Transition state	120,000 <sup>b</sup>

<sup>a</sup> Ratio of  $K_m$  values.

<sup>b</sup> Ratio of  $K_{\text{ts}}$  values.

titration calorimetry experiments ruled out the possibility of an increased affinity for the substrate.

To enhance the rate of any reaction over the rate of reaction that is observed in water, an enzyme binds the altered substrate in the transition state more tightly than it binds the substrate in the ground state ES complex (14). This difference in affinities may be achieved, at least in part, by the introduction of a local strain in the ES complex that is relieved as the ES complex proceeds toward the transition state.<sup>3</sup>

In the case of ODCase, it has been suggested that repulsive interactions between Asp-91 and the carboxylate group of OMP may produce ground state destabilization by electrostatic interactions, which are relieved in the transition state as  $\text{CO}_2$  is eliminated (5). If this enzyme acted by such a mechanism, with ground state repulsion present in the ES complex with UMP, one might expect significantly tighter binding of product OMP in which such repulsion would be absent. In fact, the affinity of the enzyme for product UMP ( $K_i = 2.0 \times 10^{-7} M$ ) is greatly exceeded by its affinity for the substrate OMP, whose  $K_m$  value ( $7 \times 10^{-7} M$ ) has been shown to be a true dissociation constant (11, 13). These affinities, combined with the present effects of active site modification, seem to indicate unequivocally that the active site binds the substrate strongly in the ground state and that their mutual affinity increases greatly as the enzyme-substrate complex progresses toward the transition state.

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<sup>3</sup> It is worth noting that the introduction of ground state destabilization elevates  $k_{\text{cat}}$  and  $K_m$  to the same extent, without enhancing  $k_{\text{cat}}/K_m$ . Most enzymes operate with the substrate at sub-saturating concentrations (15), and the introduction of ground state destabilization does not enhance the rate of reaction under these conditions.