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 O6 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, \( k_{cat} \) 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, \( k_{cat} \) was reduced by a factor of 130, and \( K_m \) 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.

The spontaneous decarboxylation of orotidine-5'-phosphate is very slow indeed (\( k_{cat} = 2.8 \times 10^{-16} \) sec\(^{-1} \)). According to the theory of absolute reaction rates, a large value of \( k_{cat}/K_m \) (for ODCase = \( 6.3 \times 10^{7} \) M\(^{-1}\) sec\(^{-1} \)) requires that the altered substrate be very tightly bound in the transition state \( K_a < 10^{-23} \) M\(^{-1} \). Moreover, a large value of \( k_{cat} \) (for ODCase = 44 s\(^{-1} \)) requires that the substrate be much less tightly bound in the ground state (1), as was recently established (\( K_m = K_a = 7 \times 10^{-7} \) M) (11, 13). From a structural standpoint, it would be undesirable 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\(_2\) 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 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, acdD) 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 \( \times 10^{-4} \) M, pH 7.2) by observing the decrease in absorbance at either 285 or 295 nm where \( \Delta \varepsilon_{295} = -1743 \) and \(-819\) cm\(^{-1}\), respectively. An alternative assay (12), monitoring the evolution of \(^{14}\)CO\(_2\) from radiolabeled OMP, was used to estimate values of \( K_m \) 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\(^{-1}\). 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 \( 10^{-3} \) M.

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Circular dichroism spectra were obtained at 25 °C using an Applied Photophysics π+ 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 O2 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 O2 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'-deoxyo-rotidine 5'-phosphate determined in a separate study.2 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 plays a critical role in transition state stabilization.

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

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

**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).
Dissecting a Charged Network at the Active Site of ODCase

The crystal structures of all ODCase-ligand complexes suggest the presence of a hydrogen bond between the ε-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 $k_{cat}$ 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 I

| Enzyme  | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ | ∆ΔG (kcal/mol) |
|---------|---------------------|-----------|---------------|----------------|
| Wild-type | 44                  | 7.0 × 10^{-7} | 6.3 × 10^{-7} | 0              |
| Q215A   | 41                  | 2.5 × 10^{-6} | 1.8 × 10^{-6} | 0.81          |
| K59A    | 0.34                | 6.4 × 10^{-4} | 5.3 × 10^{2}  | 6.8           |
| D96A    | ≪0.00023            | 8.0 × 10^{-6} | <29           | >8.6          |

*This value represents the dissociation constant of the OMP-D96A complex, determined by isothermal titration calorimetry.

Table II

| Ligand dissociation constants (mol/liter) for wild-type and mutant yeast ODCases |
|---------------------------------|------------------|------------------|------------------|
| XMP                             | K_{UMP}          | K_{XMP}          | K_{(6-AzaUMP)}   |
| Wild-type                       | 2.0 × 10^{-4}    | 7.0 × 10^{-7}    | 4.1 × 10^{-7}    |
| Q215A                           | 1.7 × 10^{-4}    | 2.5 × 10^{-6}    | 4.6 × 10^{-6}    |
| K59A                            | 3.3 × 10^{-4}    | 6.4 × 10^{-4}    | 7.3 × 10^{-4}    |

*From Ref. 17.

Table III

| Enzyme | Specific activity (μmol min^{-1} mg^{-1}) |
|--------|------------------------------------------|
| Wild-type | 90                                      |
| Q215A   | 84                                      |
| K59A    | 0.19                                    |
| D91A    | ≈0.00066                                |
| K59A    | ≈0.00018                                |
| D96A    | ≈0.00047                                |

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 $k_{cat}$ and increase the affinity of the enzyme for the substrate in the ground state. In fact, both the K59A and D96A mutant enzymes showed reduced values of $k_{cat}$ and reduced affinities for the substrate in the ground state. Similarly, both the D91A and K93A mutants were inactive, and isothermal 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.

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 CO₂ 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 ($K_i = 2.0 \times 10^{-7} \text{ M}$) is greatly exceeded by its affinity for the substrate OMP, whose $K_m$ value ($7 \times 10^{-7} \text{ M}$) 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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