Role of Enzyme−Ribofuranosyl Contacts in the Ground State and Transition State for Orotidine 5′-Phosphate Decarboxylase: A Role for Substrate Destabilization?†

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ABSTRACT: The crystal structure of yeast orotidine 5′-monophosphate decarboxylase (ODCase) complexed with the inhibitor 6-hydroxyuridine 5′-phosphate (BMP) reveals the presence of a series of strong interactions between enzyme residues and functional groups of this ligand. Enzyme contacts with the phosphoribofuranosyl moiety of orotidine 5′-phosphate (OMP) have been shown to contribute at least 16.6 kcal/mol of intrinsic binding free energy to the stabilization of the transition state for the reaction catalyzed by yeast ODCase. In addition to these enzyme−ligand contacts, active site residues contributed by both subunits of the dimeric enzyme are positioned to form hydrogen bonds with the 2′- and 3′-OH groups of the ligand's ribosyl moiety. These involve Thr-100 of one subunit and Asp-37 of the opposite subunit, respectively. To evaluate the contributions of these ribofuranosyl contacts to ground state and transition state stabilization, Thr-100 and Asp-37 were each mutated to alanine. Elimination of the enzyme's capacity to contact individual ribosyl OH groups reduced the k_{cat}/K_m value of the T100A enzyme by 60-fold and that of the D37A enzyme by 300-fold. Removal of the 2′-OH group from the substrate OMP decreased the binding affinity by less than a factor of 10, but decreased k_{cat} by more that 2 orders of magnitude. Upon removal of the complementary hydroxymethyl group from the enzyme, little further reduction in k_{cat}/K_m for 2′-deoxyOMP was observed. To assess the contribution made by contacts involving both ribosyl hydroxyl groups at once, the ability of the D37A mutant enzyme to decarboxylate 2′-deoxyOMP was measured. The value of k_{cat}/K_m for this enzyme−substrate pair was 170 M^{-1} s^{-1}, representing a decrease of more than 7.6 kcal/mol of binding free energy in the transition state. To the extent that electrostatic repulsion in the ground state can be tested by these simple alterations, the results do not lend obvious support to the view that electrostatic destabilization in the ground state enzyme−substrate complex plays a major role in catalysis.

Orotidine 5′-phosphate decarboxylase (ODCase,[1] EC 4.1.1.23) catalyzes the formation of uridine 5′-phosphate from orotidine 5′-phosphate in the final step of pyrimidine biosynthesis (Figure 1) (*1*). ODCase enhances the rate of spontaneous substrate decarboxylation by more than 17 orders of magnitude and is unusual among decarboxylating enzymes in that it functions without metals or other cofactors (*2*, *3*). Recently, the crystal structures of yeast ODCase and three bacterial enzymes in complex with different ligands, including the product UMP and two competitive inhibitors, were reported (*4*−*7*). In each of these structures, active site residues make similar contacts with functional groups of the ligand. The structure of yeast ODCase complexed with

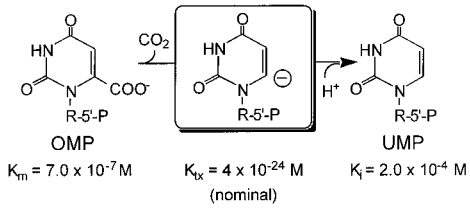

FIGURE 1: Reaction catalyzed by orotidine 5′-phosphate decarboxylase.

6-hydroxyuridine 5′-phosphate (BMP), a potential transition state analogue, shows extensive enzyme interactions with this ligand's phosphoribosyl moiety. Despite their distance from the C6 carboxylate group of OMP, enzyme−phosphate contacts have been shown to be critical for efficient substrate decarboxylation by the yeast enzyme (*8*).

Orotic acid, which lacks the phosphoribofuranosyl group, is an extremely poor substrate for enzymatic decarboxylation ($k_{cat}/K_m \leq 2.5 \times 10^{-5}$ M^{-1} s^{-1}) (*8*). Comparison of this value with k_{cat}/K_m for the natural substrate OMP (6.3×10^7 M^{-1} s^{-1}) indicates that the effective concentration of the phosphoribofuranosyl group of OMP, in stabilizing the transition state for decarboxylation, exceeds 10^8 M. In the yeast ODCase−BMP structure, the 2′-OH and 3′-OH groups of the ribofuranosyl moiety of BMP form hydrogen bond with

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[1] Abbreviations: ODCase, orotidine 5′-phosphate decarboxylase; OMP, orotidine 5′-phosphate; UMP, uridine 5′-phosphate; 6-azaUMP, 6-azauridine 5′-phosphate; XMP, xanthosine 5′-phosphate; BMP, 6-hydroxyuridine 5′-phosphate; 2′-deoxyOMP, 2′-deoxyorotidine 5′-phosphate.

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the side chains of Thr-100 and Asp-37, respectively. Thr-100 is contributed by the opposite subunit of the ODCase dimer and is located on a mobile loop that is repositioned upon ligand binding. It has been proposed that ligand interactions with Asp-37 and Thr-100 constrain the mobility of the ligand within the ODCase active site (*4*). Recent computations based on the crystal structure of the *Methanobacterium thermoautotropicum* enzyme complexed with 6-azauridine 5′-monophosphate suggested that the ligand's 2′-OH group forms a hydrogen bond with Lys-72 (equivalent to Lys-93 in yeast ODCase) in the ground state E−S complex (*5*). However, an earlier comparison of the dissociation constants of several base and nucleoside inhibitors of human UMP synthase suggested that, for this enzyme, the contribution of the ribofuranosyl moiety to ligand binding affinity is of only minor importance in stabilizing the ground state E−S complex (*9*). In the work presented here, we have explored the role in catalysis of contacts involving the ribofuranosyl OH groups, by determining the kinetic properties of mutant enzymes with alanine substituted for either Asp-37 or Thr-100. We have also synthesized 2′-deoxyOMP, assessed its activity as a substrate for wild-type and mutant enzymes, and measured the ability of other 2′-deoxy analogues to serve as inhibitors.

EXPERIMENTAL PROCEDURES

Assays for ODCase Activity and Inhibitor Binding Constants. Enzymatic decarboxylation of OMP to UMP was routinely assessed by observing the decrease in absorbance at 285 nm where $\Delta\epsilon_M = -1650$ cm^{-1} (*10*). An alternative method of assay, monitoring the evolution of $^{14}CO_2$ from ^{14}C-7-labeled OMP, was used to determine the value of K_m for recombinant wild-type yeast ODCase (*11*). Values of k_{cat} and K_m for wild-type and mutant enzymes were determined from nonlinear regression analysis of assays conducted at 25 °C in MOPS buffer ($2-10 \times 10^{-2}$ M, pH 7.2) containing varying concentrations of the substrate OMP. Wild-type and mutant enzyme concentrations were estimated from absorbance readings at 280 nm using a molar extinction coefficient of 28 830 M^{-1} cm^{-1}, estimated from the amino acid content and verified by the method of Edelhoch (*12*). Inhibitor binding constants (K_i) were determined by comparing rates of decarboxylation in the presence and absence of varying concentrations of inhibitor, using either the spectrophotometic or radioactive assay. Inhibition constants for the product UMP were determined by monitoring the decrease in absorbance at 295 nm where $\Delta\epsilon_M = -820$ cm^{-1}. Inhibitor dissociation constants were determined from double-reciprocal plots, and in each case, inhibition was competitive.

Site-Directed Mutagenesis and Protein Expression. The mutagenesis target was the *Saccharomyces cerevisiae ura3* gene of plasmid pBGM41 (*3*). Mutant genes encoding the D37A and T100A mutant enzymes were constructed by site-directed mutagenesis using Quick-Change reagents (Stratagene, Inc.) and mutagenic oligonucleotide pairs (Oligos Etc.). Following isolation of clones from the mutagenesis reaction, the complete nucleotide sequence of the mutant *ura3* gene was verified on both DNA strands with sets of overlapping primers.

Wild-type and mutant enzymes were expressed in *Escherichia coli* SS6130 (*cytR*, Δ*cdd*) as described previously (*8*). Each mutant *ura3* gene was isolated as a *Nde*I- and *Bam*HI-ended DNA fragment and inserted 3′ to the cdd promoter of cytidine deaminase expression plasmid pCDA6022. This procedure resulted in replacement of the pCDA6022 cdd gene by the mutant *ura3* DNA to yield plasmids pODCD37A and pODCT100A. Each mutant ODCase was purified as described previously (*3*). Data from electrospray mass spectrometry of the intact polypeptide, from proteolysis followed by MS/MS analysis of the fragments, and from N-terminal amino acid sequencing of proteolytic fragments indicated that each mutant enzyme contained only the desired alanine substitution.

Synthesis and Characterization of 2′-DeoxyOMP. 2′-DeoxyUMP (1.3 g) was dissolved in a mixture of pyridine (13 mL) and acetic acid (6.5 mL) with gentle stirring. The solution was cooled in an ice bath, and Br_2 (0.17 mL) was added dropwise with stirring. The reaction mixture was warmed to room temperature and stirred for 19 h, and then evaporated to dryness with repeated additions of H_2O to remove unreacted Br_2. The yield of 5-bromo-2′-deoxyUMP was greater than 90% based on ^{1}H NMR: UV λ_{max} 278 (pH 1.5), 275 nm (pH 14); ^{1}H NMR (D_2O) δ 8.24 (s, 6-H), 6.29 (t, 1′-H).

5-Bromo-2′-deoxyUMP was taken up in H_2O (5 mL), passed through a cation exchange column (Dowex H+, 1.5 cm × 15 cm), and neutralized with tetraethylammonium hydroxide. The neutral solution was evaporated to dryness, and the resulting syrup was dissolved in anhydrous DMSO (14 mL). KCN (0.18 g) and 18-crown-6 (0.05 g) were added, and the reaction mixture was stirred at room temperature for 40 h. Rotary evaporation yielded a white solid with a 70% yield of 6-cyano-2′-deoxyUMP based on ^{1}H NMR: ^{1}H NMR (D_2O) δ 6.60 (s, 5-H), 6.43 (t, 1′-H).

The crude 6-cyano-2′-deoxyUMP reaction mixture (1 mL) was evaporated to dryness, and the residue was dissolved in 3.4 M KOH (1.5 mL), stirred 4 h at 60 °C, then diluted with H_2O (20 mL), and neutralized with HCl. Acid-washed charcoal was added to the neutralized solution until all of the nucleotides were adsorbed, as indicated by UV absorbance. The charcoal was filtered from the solution, and 2′-deoxyOMP was eluted by washing with an aqueous solution of 60% ethanol and 2% (v/v) ammonium hydroxide. The washings were pooled and evaporated to a final volume of 5 mL. The remaining charcoal was removed by filtration through a 0.22 μm filter, and the filtrate was loaded onto an anion exchange column (Bio-Rad AG-1X 8 chloride form, 3 cm × 25 cm). The column was washed with 500 mL of H_2O, and 2′-deoxyOMP was eluted with a linear gradient of KCl (from 0 to 1 M), emerging at approximately 0.4 M KCl. Fractions containing 2′-deoxyOMP were pooled and desalted by treatment with acid-washed charcoal as described above. Purified 2′-deoxyOMP was characterized by NMR, UV, and IR spectroscopy: ^{1}H NMR (D_2O) δ 5.75 (s, 5-H), 5.95 (t, 1′-H); UV λ_{max} 266 nm (pH 7.0); IR KBr (COO^-) 1630, 1406 cm^{-1}.

Enzymatic decarboxylation of 2′-deoxyOMP was monitored at pH 7.2 using the spectrophotometric assay described above. The K_m value of 2′-deoxyOMP was estimated from the competitive inhibition of OMP decarboxylation observed in the presence of increasing concentrations of 2′-deoxyOMP. The value of k_{cat} was determined from the rate of decarboxylation of 2′-deoxyOMP at concentrations of the trun-

Table 1: Ligand Dissociation Constants for Wild-Type Yeast ODCase

	K_i(base) (M)	K_i(side) (M)	K_i(base)/ K_i(side)	K_i (M)
uracil	1.6×10^{-2}	2.3×10^{-2}	0.7	
orotate	9.5×10^{-3}	1.4×10^{-3}	6.8	
xanthine	$>6.2 \times 10^{-4}$	5.3×10^{-4}	>1.2	
6-azauracil	5.9×10^{-3}	1.2×10^{-2}	0.5	
barbiturate	6.0×10^{-6}	9.3×10^{-6}	0.6	
ribose				3.7×10^{-2}
UMP				2.0×10^{-4}
2′-deoxyUMP				2.6×10^{-4}
ribose 5′-phosphate				8.1×10^{-5}
2′-deoxyribose 5′-phosphate				7.0×10^{-4}

Table 2: Kinetic Properties of Wild-Type and Mutant Yeast ODCases

	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G^a$ (kcal/mol)
wild type				
OMP	44	7.0×10^{-7}	6.3×10^{7}	(0)
2′-deoxyOMP	0.15	5.4×10^{-6}	2.8×10^{4}	4.6
D37A				
OMP	0.9	4.2×10^{-6}	2.1×10^{5}	3.4
2′-deoxyOMP	0.0017	1.0×10^{-5}	1.7×10^{2}	7.6
T100A				
OMP	7.2	6.7×10^{-6}	1.1×10^{6}	2.4
2′-deoxyOMP	0.16	5.2×10^{-6}	3.1×10^{4}	4.5

[a] $\Delta\Delta G = -RT \ln\{[k_{cat}/K_m(\text{mutant})]/[k_{cat}/K_m(\text{wild type})]\}$.

cated substrate analogue ranging from 6.6×10^{-5} to 2.2×10^{-4} M.

RESULTS

Properties of Wild-Type ODCase. The apparent dissociation constants measured for substrates and competitive inhibitors with wild-type ODCase at pH 7.4 are given in Table 1. The kinetic parameters k_{cat}, K_m, and k_{cat}/K_m determined for the enzymatic decarboxylation of OMP and 2′-deoxyOMP by wild-type yeast ODCase are summarized in Table 2.

Properties of Mutant Enzymes. Expression of D37A and T100A mutant enzymes in *E. coli* yielded large quantities of stable, soluble, recombinant mutant proteins. Mass spectrometry and N-terminal sequence analysis verified that each mutant protein was expressed with only the expected amino acid substitution. Table 2 shows the k_{cat} and K_m values for each mutant enzyme, determined by nonlinear regression analysis of data collected from activity assays with each mutant enzyme. The k_{cat} values for both the D37A and T100A enzymes were reduced, while the K_m value for each mutant enzyme was elevated, compared with the corresponding values for the wild-type decarboxylase. The overall decrease in catalytic efficiency, k_{cat}/K_m, resulting from the Asp-37 → Ala and Thr-100 → Ala substitutions and the corresponding free energy difference ($\Delta\Delta G$) between each mutant and wild-type enzyme are presented in Table 2.

The dissociation constants for UMP, XMP, and 6-azaUMP, derived from analysis of data for these competitive inhibitors, are shown in Table 3. Values representing the maximal dissociation constant of the substrate in the transition state (K_{tx}) for wild-type and mutant enzymes were calculated from the ratio of k_{non} for the decarboxylation of 1-methylorotate (2.8×10^{-16} s^{-1}) to the value of k_{cat}/K_m for each enzyme (*2*).

DISCUSSION

Contributions of the Ribofuranosyl Group to Equilibrium Binding Affinity. Earlier, we reported the contributions of individual substrate binding determinants to transition state stabilization by the bifunctional enzyme human UMP synthase (*9*). With the aid of new structural information concerning the active sites of the monofunctional ODCases from yeast and bacteria (*4*−*7*), we undertook a systematic examination of the contributions made by each substrate or inhibitor binding determinant to transition state stabilization or ligand binding by the yeast enzyme. Comparison of the K_i values of orotidine and xanthosine with those of orotic acid and xanthine indicates only a slight increase in binding affinity that can be attributed to the ribofuranosyl group of these nucleosides (Table 1). Similarly, removal of the 2′-OH group from UMP and ribose 5′-phosphate resulted in only a modest decrease in the binding affinity of both ligands (Table 1). Addition of a ribofuranosyl substituent to uracil, 6-azauracil, and barbituric acid was found to weaken binding affinity slightly. Considered as a separate molecule, ribose is a weak inhibitor of yeast ODCase, with a K_i value of 3.7×10^{-2} M. From the absence of any apparent contribution of substituent ribose to the binding affinity of nucleosides, it seems reasonable to infer either that substituent ribose remains exposed to solvent in the enzyme−nucleoside complex or that solvent water is able to compete effectively with the ribose portion of the nucleoside for a place on the enzyme. The contribution of the ribofuranosyl moiety becomes manifest only in the context of the nucleotide, in which it seems to function as a connecting link between two major substrate binding determinants, the pyrimidine ring and the phosphate substituent. This conclusion is supported by the results of enzyme and substrate modifications described below.

Contributions of the 3′-OH Group to Catalysis. For yeast ODCase, the K_m value of OMP has been shown to be a true dissociation value (*15*). In the yeast crystal structure, Asp-37 makes a single hydrogen bond with the 3′-OH group of the ribofuranosyl group of BMP (Figure 2). The effects of eliminating this enzyme−ligand contact on k_{cat}, K_m, and k_{cat}/K_m are summarized in Table 2. Removal of the carboxymethyl group of Asp-37 resulted in a decrease in k_{cat} from 44 to 0.9 s^{-1} and an increase in K_m from 7.0×10^{-7} to 4.2×10^{-6} M. Together, these altered kinetic parameters indicate that the binding affinity of the mutant enzyme for the altered substrate in the transition state is reduced 300-fold compared

Table 3: Dissociation Constants for Enzyme−Ligand Complexes for Wild-Type and Mutant Yeast OD Cases

	K_i(UMP) (M)	K_m(OMP) (M)	K_i(XMP) (M)	K_i(6-azaUMP) (M)	K_{tx} (M)
wild type	2.0×10^{-4}	7.0×10^{-7}	4.1×10^{-7}	6.4×10^{-8}	4.4×10^{-24}
D37A	2.1×10^{-4}	4.2×10^{-6}	3.9×10^{-5}	7.7×10^{-6}	1.3×10^{-21}
T100A	3.7×10^{-4}	6.7×10^{-6}	1.2×10^{-5}	9.4×10^{-6}	2.6×10^{-22}

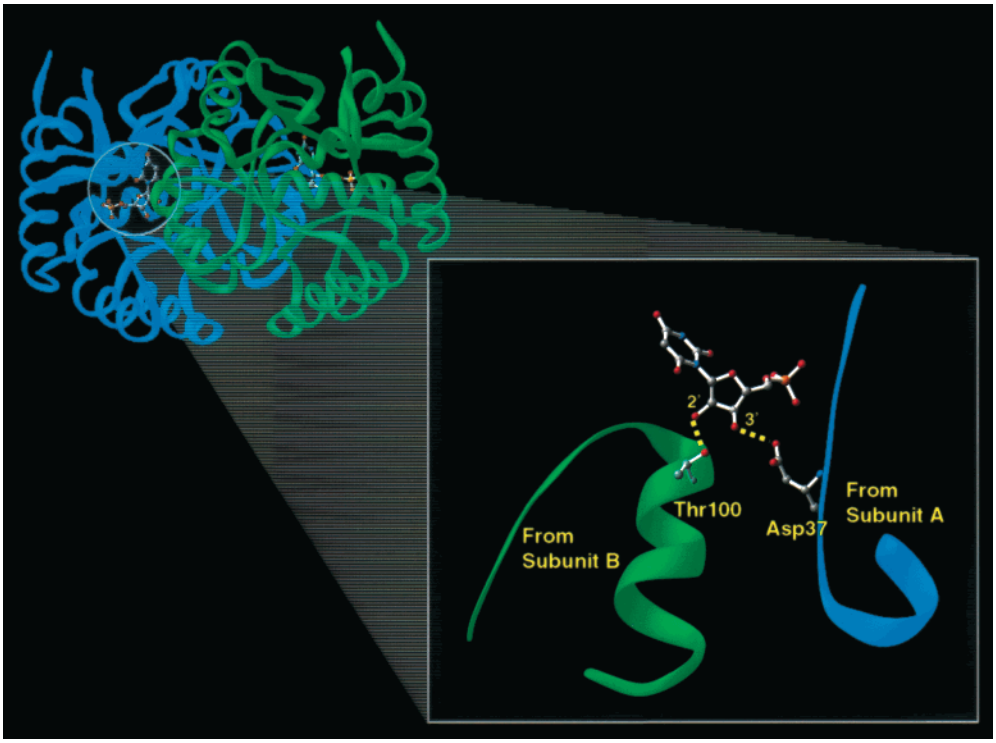

FIGURE 2: Structure of the yeast ODCase dimer in complex with 6-hydroxyuridine 5′-phosphate (background), showing interactions between Thr-100 and Asp-37 and the 2′- and 3′-OH groups of the ribofuranosyl moiety of this ligand (foreground).

Table 4: Relative Contributions of Enzyme−Ribofuranosyl Contacts to Substrate, Inhibitor, and Transition State Binding Affinity

ligand	K_i(D37A)/K_i(wild type)	K_i(T100A)/K_i(wild type)
UMP	1.1	1.9
OMP	6[a]	9.6[a]
XMP	95	29
6-azaUMP	120	147
transition state	295	59

[a] Determined from substrate K_m values, which represent true dissociation constants for the enzyme−substrate complex (see refs *8* and *15*).

with that of the wild-type enzyme. Removal of the carboxymethyl group of Asp-37 only slightly altered the binding affinity of the product UMP but did decrease the binding affinity of other ligands (substrate OMP, the inhibitors XMP and 6-AzaUMP, and the altered substrate in the transition state) (Table 4). This difference seems to be consistent with the increasing importance of phosphoribofuranosyl contacts as the reaction progresses toward the transition state (*8*).

Contributions of the 2′-OH Group to Catalysis. The crystal structure of the yeast ODCase−BMP complex shows that the active site is composed of amino acid residues from both monomers of the catalytically active ODCase dimer (Figure 2). Upon ligand binding, the side chains of Thr-100 and Asp-96 from the opposite subunit reach into the ODCase active site and establish bonding contacts with the 2′-OH group of BMP. The effect of eliminating the interaction between Thr-100 and the 2′-OH group of the ligand was less profound than the effects described above for the loss of a hydrogen bond between Asp-37 and the 3′-OH group. The k_{cat} value for the T100A enzyme was reduced from 44 to 7.2 s^{-1}, while K_m increased from 7.0 × 10^{-7} to 6.7 × 10^{-6} M (Table 2), resulting in a 2.4 kcal/mol reduction in the binding free energy of the altered substrate in the transition state. Removal of the Thr-100 side chain produced only modest decreases in the binding affinities of UMP, XMP, and 6-azaUMP (Tables 3 and 4).

Of special interest is the observation that the 2′-OH group is the sole site of contact between the ligand and residues contributed by the opposite subunit (*4*−*7*). The 2′-OH group of OMP has been postulated to form a hydrogen bond to Lys-72 of the *M. thermoautotropicum* enzyme in the ground state complex (*5*). In the experiments described here, removal of the side chain of Thr-100 reduced k_{cat}/K_m for OMP by 60-fold. To assess the contribution of contacts with the 2′-OH group to ground state and transition state affinity in a different way, we synthesized 2′-deoxyOMP and tested the ability of yeast ODCase to act on this truncated substrate analogue. For wild-type ODCase, the K_m value of 2′-deoxyOMP was 5.4 × 10^{-6} M, 8-fold higher than the value for the natural substrate, and k_{cat} was reduced by more than 2 orders of magnitude, from 44 to 0.15 s^{-1} (Table 2). Thus, truncation of the 2′-OH group of the substrate OMP reduced k_{cat}/K_m by more than 2200-fold, corresponding to a 4.6 kcal/mol reduction of the binding free energy of the altered substrate in the transition state. These results indicate that enzyme interactions with the 2′-OH group are important for substrate turnover, but do not contribute significantly to the enzyme's affinity for the substrate in the ground state.

When *both* Thr-100 and the substrate's 2′-OH group were removed, the k_{cat} value for 2′-deoxyOMP was 0.16 s^{-1} and the K_m value was 5.2 × 10^{-6} M. These values resemble the k_{cat} and K_m values observed for the action of wild-type ODCase on 2′-deoxyOMP. The difference between the catalytic consequences of Thr-100 mutagenesis and removal of the substrate's 2′-OH group may arise from the fact that the 2′-OH group also forms a hydrogen bond to Asp-96, which would be eliminated by removal of the 2′-OH group from the substrate OMP.

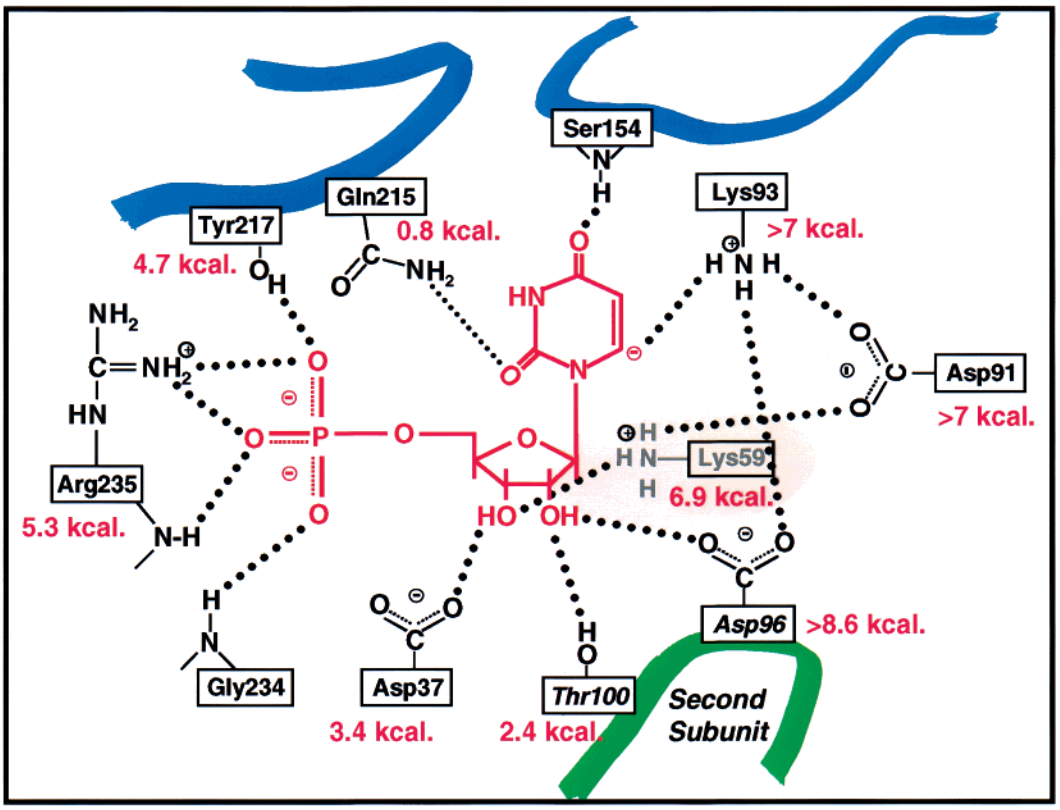

FIGURE 3: Effect ($\Delta\Delta G$) of single alanine mutations on the ability of ODCase to stabilize the altered substrate in the transition state for OMP decarboxylation. The sum of the individual mutations, shown in red adjacent to the mutated residue, amounts to more than 45 kcal/mol of binding free energy. The shaded oval surrounding Lys-59 is intended to indicate that this residue approaches the ligand from below. The colored ribbons represent polypeptide regions of the ODCase subunits that move in response to binding of the ligand.

To examine the consequences of removing enzyme contacts with both the 2′-OH and 3′-OH groups of substrate OMP, we measured the ability of the D37A mutant enzyme to catalyze the decarboxylation of 2′-deoxyOMP. This operation removes three enzyme−ligand interactions, one mediated by the 3′-OH group of OMP and two mediated by the 2′-OH group. Table 2 shows that the k_{cat} value for the D37A enzyme acting on 2′-deoxy OMP was 0.0017 s^{-1} while the K_m value was 1.0×10^{-5} M. The combined effect of removing both the Asp-37 side chain and the 2′-OH group of OMP is to reduce the free energy of binding of the altered substrate in the transition state by 7.6 kcal/mol (Table 2).

When several enzyme−ligand interactions are disrupted by enzyme mutation and/or ligand truncation, it is of interest to ask whether the combined effect of individual mutations is equal to the overall change in binding free energy observed when multiple interactions are eliminated simultaneously (*13*). The availability of both D37A mutant ODCase and 2′-deoxyOMP allows such a comparison to be made for the reaction catalyzed by yeast ODCase. The individual changes in transition state binding free energy for the Asp-37 → Ala and 2′-OH truncations are 3.4 and 4.6 kcal/mol, respectively. The combined effect of removing both of these groups, as reflected in the comparison of the value of k_{cat}/K_m for wild-type ODCase with the value of k_{cat}/K_m for D37A ODCase acting on 2′-deoxyOMP, is 7.6 kcal/mol (Table 2). When this value is compared with the sum of the effects of the individual D37A and 2′-deoxyOMP modifications (8.0 kcal/mol), their effects are seen to be nearly additive. This near additivity is somewhat surprising considering the extreme departures from additivity observed in our earlier study of the effects of modifying enzyme interactions with the phosphoryl group (*8*).

These experiments, which complete our survey of the effects on catalysis resulting from alanine substitution for each active site residue whose side chain makes direct contact with 6-hydroxyuridine 5′-phosphate (BMP) in the crystal structure, are summarized in Figure 3. Removal of electrostatic interactions between the enzyme and the substrate's phosphoryl group (*8*), or disruption of the charged network of residues located near the reactive carboxyl group of OMP (*16*), is seen to result in drastic losses of activity.

Because of the proximity of Asp-91 to the expected location of the substrate's reactive carboxyl group, ground state destabilization has been invoked to explain the remarkable catalytic proficiency of ODCase (*5*−*7*, *14*). If repulsive interactions between Asp-91 and the substrate's carboxylate group drove this reaction, then one might expect UMP (lacking that carboxylate group) to be bound with greater affinity than OMP, but that is not the case (Table 5). One might also expect that any mutation that relieved the ground state destabilization would tend to increase the enzyme's affinity for the substrate in the ground state. Instead, mutation is found to reduce k_{cat} and *increase* the value of K_m,[2] for each of the seven alanine mutants whose affinities have been measured (Table 5).

As is the case for every enzyme reaction, enzyme−substrate interactions are far less favorable in the ground state

[2] Earlier work has shown that, for the wild-type enzyme, K_m represents the dissociation constant of the enzyme−substrate complex (*8*, *15*).

Table 5: Summary of the Kinetic Properties of Wild-Type and Mutant ODCases

enzyme	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G$ (kcal/mol)	ref
wild type	44	7.0×10^{-7}	6.3×10^{7}	(0)	*8*
Q215A	41	2.5×10^{-6}	1.6×10^{7}	0.8	*16*
T100A	7.2	6.7×10^{-6}	1.1×10^{6}	2.4	this work
D37A	0.9	4.2×10^{-6}	2.1×10^{5}	3.4	this work
Y217A	2.0	9.4×10^{-5}	2.1×10^{4}	4.7	*8*
R235A	0.42	4.9×10^{-5}	8.6×10^{3}	5.3	*8*
K59A	0.34	6.4×10^{-4}	5.3×10^{2}	6.9	*16*
D91A	nd[a]	nd	nd	>7.0	*16*
K93A	nd	nd	nd	>7.0	*16*
D96A	<0.00023	8.0×10^{-6}	<29	>8.6	*16*

[a] nd, not determined due to a lack of measurable activity (see ref *16*).

than in the transition state. To the extent that electrostatic repulsion in the ground state can be tested by these simple alterations, the results do not lend obvious support to the view that electrostatic destabilization in the ground state enzyme−substrate complex plays a major role in catalysis. However, because of the extreme departures from additivity of interactions that have been observed in ligand interactions at the active site of ODCase (8), any interpretation that assumes simple subtractive effects of mutation is likely to be an oversimplification.

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