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Contribution of Enzyme−Phosphoribosyl Contacts to Catalysis by Orotidine 5′-Phosphate Decarboxylase†

Brian G. Miller,‡ Mark J. Snider,‡ Steven A. Short,*,§ and Richard Wolfenden*,‡

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260, and Department of Molecular Sciences, GlaxoWellcome, 5 Moore Drive, Research Triangle Park, North Carolina 27709

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ABSTRACT: The crystal structure of the complex formed between recombinant yeast orotidine 5′-phosphate decarboxylase and the competitive inhibitor 6-hydroxyuridine 5′-phosphate reveals the presence of four hydrogen bonds between active site residues Tyr-217 and Arg-235 and the phosphoryl group of this inhibitor. When Tyr-217 and Arg-235 are individually mutated to alanine, values of k_{cat}/K_m are reduced by factors of 3000- and 7300-fold, respectively. In the Y217A/R235A double mutant, activity is reduced more than 10^7-fold. Experiments with highly enriched [^{14}C]orotic acid show that when ribose 5′-phosphate is deleted from substrate orotidine 5′-phosphate, k_{cat}/K_m is reduced by more than 12 orders of magnitude, from 6.3×10^7 M^{-1} s^{-1} for OMP to less than 2.5×10^{-5} M^{-1} s^{-1} for orotic acid. Activity toward orotate is not "rescued" by 1 M inorganic phosphate. The K_i value of ribose 5′-phosphate, representing the part of the natural substrate that is absent in orotic acid, is 8.1×10^{-5} M. Thus, the effective concentration of the 5′-phosphoribosyl group, in stabilizing the transition state for enzymatic decarboxylation of OMP, is estimated to be $>2 \times 10^8$ M, representing one of the largest connectivity effects that has been reported for an enzyme reaction.

In the final step of pyrimidine biosynthesis, orotidine 5′-phosphate (OMP)[1] is decarboxylated to form uridine 5′-phosphate (UMP) (Figure 1) (*1*). In yeast and lower eukaryotes, orotidine 5′-phosphate decarboxylase (ODCase, EC 4.1.1.23) exists as a distinct protein molecule, whereas in humans, this enzyme is joined to orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) in a bifunctional enzyme complex (*2*). ODCase is an unusually proficient catalyst. In neutral aqueous solution, the spontaneous decarboxylation of orotic acid derivatives proceeds extremely slowly ($t_{1/2} = 78 \times 10^6$ years). Primary amines do not enhance the rate of this reaction even at high concentrations, and replacement of water by other solvents has surprisingly little effect on the rate of reaction. At the active site of yeast OMP decarboxylase, the same reaction takes place with a rate constant of 39 s^{-1}, implying that the enzyme develops an extremely high affinity for the altered substrate in the transition state (*3*). Unlike previously characterized decarboxylases that employ metals or other cofactors, ODCase acts without cofactors, by a novel mechanism that may involve stabilization of a carbanionic intermediate by an active site lysine residue (*4*).

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* To whom correspondence should be addressed: Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260.

‡ University of North Carolina.

§ GlaxoWellcome.

[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.

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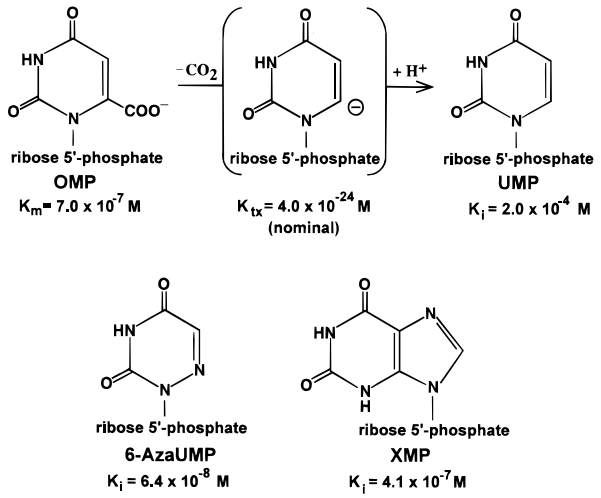

FIGURE 1: Reaction catalyzed by yeast OMP decarboxylase and the structures of two competitive inhibitors, 6-azauridine 5′-phosphate and xanthosine 5′-phosphate.

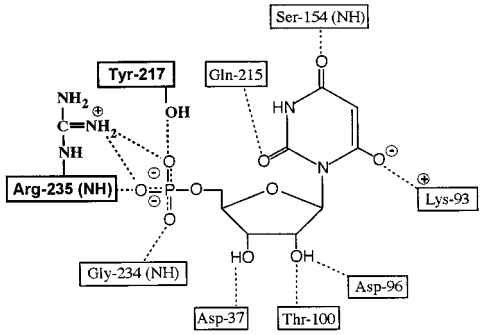

FIGURE 2: Interactions between ODCase active site residues and the postulated transition state analogue, 6-hydroxyuridine 5′-phosphate (BMP), as revealed by the crystal structure of the inhibitory complex.

We would like to understand the momentary change in affinity that occurs during catalysis, and how such high affinity is achieved in the transition state. That question can be addressed experimentally by removing individual ligand−enzyme interactions, either by mutating the enzyme's active site or by varying the structures of substrates and inhibitors. Crystal structures recently have been determined for recombinant ODCase from *Saccharomyces cerevisiae* (*5*) and from three prokaryotes (*6*−*8*). The crystal structure of the yeast enzyme, in complex with the postulated transition state analogue 6-hydroxyuridine 5′-phosphate (BMP), reveals the presence of 11 hydrogen bonds between active site residues and the ligand (Figure 2). Four of these hydrogen bonds result from contacts between the 5′-phosphoryl group of BMP and two amino acids, Tyr-217 and Arg-235. Comparison of the structures of the free and BMP-inhibited enzymes reveals that Tyr-217 is located within a loop region of the protein that envelops the phosphoryl group of the ligand, forming a protein barrier between the ligand and solvent. In contrast to the complete repositioning of Tyr-217, the position of only the side chain of Arg-235 is altered by the binding of a ligand at the active site (*5*).

Although the 5′-phosphoryl group of the substrate is distant from the site of substrate decarboxylation, earlier experiments on the human enzyme suggested a major role for this phosphoryl group in substrate activity (*9*). In the work described here, we examined the effects of mutating Tyr-217 and Arg-235 to alanine, to assess the contributions made by these residues to substrate binding affinity in the ground state and transition state. In a complementary operation, we truncated the structures of substrates and inhibitors to determine the apparent contributions made by substrate binding determinants to transition state stabilization by yeast ODCase.

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 (*11*), was used to determine the value of K_m for recombinant wild-type yeast ODCase. Assays were conducted at 25 °C in MOPS buffer ($2-10 \times 10^{-2}$ M, pH 7.4) containing varying concentrations of substrate OMP. Concentrations of the wild-type and mutant enzyme 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*). Binding constants (K_i) for competitive inhibitors, including orotate and orotidine, were obtained by conventional methods, by comparing rates of decarboxylation in the presence and absence of varying concentrations of inhibitor, using either the spectrophotometric or radioactive assay. Inhibition constants for product UMP were determined by monitoring the decrease in absorbance at 295 nm where $\Delta\epsilon_M = -820$ cm^{-1}.

Site-Directed Mutagenesis and Protein Expression. The mutagenesis target was the *S. cerevisiae ura3* gene of plasmid pBGM41 (*13*). Mutant genes encoding Y217A, R235A, and Y217A/R235A enzymes were constructed by site-directed mutagenesis using Quick-Change reagents (Stratagene, Inc.) and mutagenic oligonucleotide pairs (Oligos Etc.). Following isolation of clones from each mutagenesis reaction, the complete nucleotide sequence of the mutated *ura3* gene was verified on both DNA strands with sets of overlapping primers.

Wild-type and enzyme mutants were expressed in *Escherichia coli* SS6130 (*cytR*, Δ*cdd*) as described previously (*13*). Briefly, each mutated *ura3* gene was isolated as a *Nde*I, *Bam*HI-ended DNA fragment that was inserted 3′ to the *cdd* promoter of cytidine deaminase expression plasmid pCDA6022. These manipulations resulted in replacement of the pCDA6022 *cdd* gene by the mutant *ura3* DNA, yielding plasmids pODCY217A, pODCR235A, and pODCY217A/R235A.

Each mutant ODCase was purified from bacteria that had been grown at 25 °C for 16−18 h in 2XYT (*14*) supplemented with Vogel and Bonner salts (*15*), 0.5% yeast extract, uracil (100 μg/mL), and ampicillin (150 μg/mL). Cell extracts were prepared from frozen cells, and each mutant protein was purified by anion exchange chromatography in Tris-HCl (5.0×10^{-2} M, pH 7.0) containing dithiothreitol (5.0×10^{-3} M) and glycerol (20% w/v) as described previously (*13*). Following chromatography, the purity of each protein was found to be greater than 95% by SDS−PAGE analysis. Electrospray mass spectrometry of purified ODCase mutant proteins demonstrated that each mutant protein contained

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

	K_i(base) (M)	K_i(nucleoside) (M)	K_i(nucleotide) (M)	K_i(side)/K_i(tide)	K_i (M)
uracil	1.6×10^{-2}	2.3×10^{-2}	2.0×10^{-4}	1.2×10^{3}	
orotate	9.5×10^{-3}	1.4×10^{-3}	7.0×10^{-7} [a]	2.0×10^{3}	
xanthine	$>6.2 \times 10^{-4}$	5.3×10^{-4}	4.1×10^{-7}	1.3×10^{3}	
6-azauracil	5.9×10^{-3}	1.2×10^{-2}	6.4×10^{-8}	1.9×10^{5}	
barbiturate	6.0×10^{-6}	9.3×10^{-6}	8.8×10^{-12} [b]	1.1×10^{6}	
ribose					3.7×10^{-2}
phosphate					7.3×10^{-3}
ribose 5′-phosphate					8.1×10^{-5}

[a] K_m for substrate OMP as determined via the radioactive assay described in Experimental Procedures. [b] From ref *25*.

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)
OMP				
wild-type	44	7.0×10^{-7}	6.3×10^{7}	(1.0)
Y217A	2.0	9.4×10^{-5}	2.1×10^{4}	4.7
R235A	0.42	4.9×10^{-5}	8.6×10^{3}	5.3
orotic acid				
wild-type	$<2.6 \times 10^{-3}$	9.5×10^{-3}	$<2.5 \times 10^{-5}$	>16.9

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

only the desired alanyl substitution(s) at position 217 and/or 235.

Decarboxylation of Orotic Acid. The ability of recombinant yeast ODCase to catalyze the decarboxylation of orotic acid was tested by incubating high concentrations of enzyme (1.0×10^{-4} M in subunits) with uniformly ^{14}C-labeled orotic acid (250 mCi/mmol, 2.0×10^{-4} M) in MOPS buffer (5.0×10^{-2} M, pH 7.2). Following incubation for 14.5 h at 25 °C, the reactions were quenched by addition of 6 M HCl, and the $^{14}CO_2$ produced by decarboxylation of orotic acid was trapped on KOH-soaked filter paper. Activity assays before and after incubation at 25 °C indicated no significant loss of activity over the time course of the reactions.

Viscosity Effects on Enzyme Activity. Effects of viscosity on k_{cat} for wild-type yeast ODCase were determined in the presence of increasing concentrations of the disaccharide trehalose, using the spectrophotometric assay described above. To determine the effects of added trehalose on k_{cat}/K_m, the competitive inhibitor 6-AzaUMP (6.4×10^{-5} M) was added, increasing the apparent K_m of OMP from 7.0×10^{-7} to 1.2×10^{-3} M. Relative viscosities were determined at 25 °C using a Cannon-Fenske kinematic viscometer.

RESULTS

Properties of Wild-Type ODCase. Apparent dissociation constants measured for substrates and competitive inhibitors with wild-type ODCase at pH 7.4 are shown in Table 1. Table 2 summarizes the second-order rate constants (k_{cat}/K_m) calculated for decarboxylation of OMP and orotic acid by wild-type yeast ODCase. Increasing concentrations of trehalose, at relative viscosities as high as 6, were found to have no significant effect on k_{cat} or k_{cat}/K_m for yeast ODCase. Using uniformly labeled orotic acid with high specific activity (250 mCi/mmol), no significant decarboxylation of orotic acid was detected after 14.5 h in the presence of recombinant ODCase at concentrations up to 10^{-4} M in subunits. Activity assays before and after incubation at 25 °C indicated no significant loss of activity during the course of these measurements, setting an upper limit of 2.5×10^{-5} M^{-1} s^{-1} on k_{cat}/K_m for the decarboxylation of orotic acid. Attempts to rescue orotic acid decarboxylation by the addition of inorganic phosphate (1 M) proved to be unsuccessful. The enzyme's inability to decarboxylate orotic acid is not due to an inability of ligand to bind to yeast ODCase, since the enzyme−orotic acid complex is characterized by a K_i value of 9.5×10^{-3} M (Table 1).

Properties of Mutant Enzymes. Expression of Y217A, R235A, and Y217A/R235A mutant enzymes in *E. coli* yielded soluble, stable recombinant mutant proteins. Each protein, when analyzed by N-terminal sequencing and by electrospray mass spectrometry, contained only the genetic alterations specified by the mutant *ura3* coding sequences, verifying the faithful translation of each gene during bacterial expression. When the kinetic parameters k_{cat} and K_m were calculated for each mutant enzyme, the Y217A and R235A proteins exhibited significant reductions in k_{cat}, and also a marked increase in K_m compared with values obtained for the wild-type enzyme (Table 2). The combination of both mutations in a single protein reduced the activity of the Y217A/R235A enzyme below the limits of detection ($k_{cat}/K_m < 2$ M^{-1} s^{-1}). The decreases in k_{cat}/K_m resulting from the Y217A and R235A substitutions are shown in Table 2 as a change in the free energy difference between the substrate in the ground state and the altered substrate in the transition state ($\Delta\Delta G$).

The consequences of eliminating specific ligand−enzyme contacts were also examined by determining inhibitor dissociation constants for UMP, 6-AzaUMP, and BMP with each mutant enzyme (Table 3). K_i values could not be determined for the Y217A/R235A protein because its activity was not measurable.

DISCUSSION

Contributions of the Phosphoryl Group to Equilibrium Binding Affinity. Inorganic phosphate shows a relatively weak affinity for the active site ($K_i = 7.3 \times 10^{-3}$ M), but when a phosphoryl group is incorporated into the structure of a nucleotide, as in OMP, UMP, 6-AzaUMP, XMP, and BMP, its influence becomes highly significant (Table 1).

Moreover, the magnitude of the phosphoryl group's apparent contribution to binding affinity increases with increasing affinity of the nucleotide to which it is attached. For example, comparison of the dissociation constant of UMP with that of uridine indicates a 120-fold increase in binding affinity that can be attributed to the presence of the phosphoryl group. A much larger effect (10^6-fold) is observed if the dissociation constant of the postulated transition state analogue BMP is compared with that of its nucleoside

Table 3: Ligand Dissociation Constants for Wild-Type and Mutant Yeast ODCases

	K_i(UMP) (M)	K_m(OMP) (M)	K_i(6-AzaUMP) (M)	K_i(BMP) (M)	K_{tx} (M)
wild-type	2.0×10^{-4}	7.0×10^{-7}	6.4×10^{-8}	8.8×10^{-12}	4.4×10^{-24}
Y217A	3.3×10^{-3}	9.4×10^{-5}	1.4×10^{-4}	1.8×10^{-8}	1.3×10^{-20}
R235A	1.8×10^{-3}	4.9×10^{-5}	8.7×10^{-5}	2.3×10^{-8}	3.3×10^{-20}

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

ligand	K_i(Y217A)/K_i(wild-type)	K_i(R235A)/K_i(wild-type)
UMP	17	9
OMP	130[a]	70[a]
6-AzaUMP	2200	1400
BMP	2000	2600
transition state	3000	7500

[a] These values represent differences in K_m for substrate OMP.

analogue 6-hydroxyuridine. These findings suggest that contacts between enzyme residues and the phosphoryl group of the substrate become increasingly important when the transition state is approached. That possibility is supported by the results of site-directed enzyme modification described below.

Contributions of the Phosphoryl Group to Catalysis. In the crystal structure of yeast ODCase, Tyr-217 and Arg-235 form most of the contacts between the enzyme and the phosphoryl group of BMP (*5*). Table 2 summarizes the effects of mutation of these residues on k_{cat}, K_m, and k_{cat}/K_m. In each case, removal of enzyme−phosphoryl contacts has a substantial effect on the mutant enzyme's kinetic parameters. These mutations also alter the binding affinities of product UMP, and inhibitors 6-AzaUMP and BMP, and the calculated affinity of the altered substrate in the transition state (Table 3). As was inferred above from the relative binding affinities of ligands, the effects of mutation suggest that the relative benefits of contacts with the phosphoryl group increase as the ligand's affinity approaches the affinity generated in the transition state. The ratio of the K_i for UMP inhibition of wild-type ODCase to the corresponding K_i for UMP inhibition of either Y217A or R235A ODCase indicates a 10-fold increase in binding affinity that can be attributed to interactions with the phosphoryl group of UMP (Table 4). In contrast, comparison of the differences in binding affinity ($1/K_{tx}$) of the altered substrate in the transition state among wild-type, Y217A, and R235A ODCase reveals a more than 1000-fold increase in affinity resulting from contacts with the phosphoryl group (Table 3). The factors by which the enzyme's affinities for 6-AzaUMP and BMP are decreased upon substituting alanine for Tyr-217 roughly match the factor (3000-fold) by which the enzyme's affinity for the altered substrate in the transition state is decreased as a result of the same substitution (Table 4). The factors by which the affinity of 6-AzaUMP and BMP are decreased upon substituting alanine for Arg-235 do not differ greatly with the decrease in affinity (7500-fold) estimated for the altered substrate in the transition state. This would seem to be understandable if interactions between Tyr-217 and Arg-235 and the phosphoryl group of 6-AzaUMP and BMP resembled the interactions of these residues with the phosphoryl group of the altered substrate in the transition state, in terms of the magnitude of the attractive forces that are generated by these contacts.

Connectivity Effects in the Transition State for OMP Decarboxylation. Transition state affinity would be underestimated if physical binding of the substrate were to limit the value of k_{cat}/K_m, or if product release were to limit the value of k_{cat}.[2] Therefore, it is of interest that both k_{cat}/K_m and k_{cat} for the action of yeast ODCase on OMP are insensitive to changing viscosity. These observations, and the substantial ^{13}C kinetic isotope effect reported for OMP labeled with ^{13}C at the carboxyl group (*19*), indicate that k_{cat}/K_m for OMP is not diffusion-limited, and that k_{cat} is not limited by product release. Thus, k_{cat}/K_m is likely to represent the equilibrium constant for conversion of the free enzyme and substrate to a chemical transition state in which decarboxylation is in progress.

The value of k_{cat}/K_m for the action of yeast ODCase on OMP exceeds k_{cat}/K_m for its action on orotate by a factor of more than 10^{12}. In earlier work, the bifunctional UMP synthase purified from human placenta was found to exhibit a very low k_{cat}/K_m value for decarboxylation of orotate. Using a relatively insensitive spectrophotometric assay, the rate of decarboxylation of orotate was found to be decreased by a factor of (5×10^8)-fold compared with the same enzyme's k_{cat}/K_m for OMP, and at those levels, the presence of a contaminating orotate decarboxylase could not be ruled out. In this work on the recombinant enzyme from yeast, sensitivity was enhanced using orotate labeled with ^{14}C at the 6-carboxylate group. The value of k_{cat}/K_m for orotate was found to have decreased by a factor of at least 10^{12}-fold compared with the k_{cat}/K_m for OMP. The magnitude of this enzyme's ability to discriminate against an analogue of its natural substrate seems especially remarkable considering that orotate is smaller than the natural substrate and can presumably gain access to the active site.

At first glance, the vastly greater apparent stability of the transition state for enzymatic decarboxylation of OMP might be attributed to direct (intramolecular) participation of the 5′-phosphoryl group in catalysis. However, no obvious mechanism appears to be available for intramolecular participation of the phosphoryl group in stabilizing an anionic

[2] The equation $K_{tx} = k_{non}/(k_{cat}/K_m)$ assumes that k_{cat}/K_m provides a measure of the equilibrium constant for conversion of the free enzyme and substrate to the enzyme−substrate complex in the transition state for chemical transformation of the substrate. There appear to be two kinds of exceptions (*16*), both of which would be expected to lead to underestimation of transition state affinity (or overestimation of K_{tx}). (A) If k_{cat}/K_m is limited by diffusional encounter of the enzyme and substrate, rather than by chemical transformation of the substrate, then the enzyme's ability to stabilize the transition state for chemical transformation of the substrate will have been underestimated. Enzymes of this kind, typified by triosephosphate isomerase (*17*) and alkaline phosphatase (*18*), show k_{cat}/K_m values that vary with solvent viscosity. (B) If k_{cat} is limited by product release, as is often the case for dehydrogenases, then the enzyme is capable of stabilizing the transition state for chemical transformation of the substrate to a greater extent than K_{tx} would appear to indicate. Accordingly, the enzyme's ability to stabilize the transition state for chemical transformation of the substrate will once again have been underestimated.

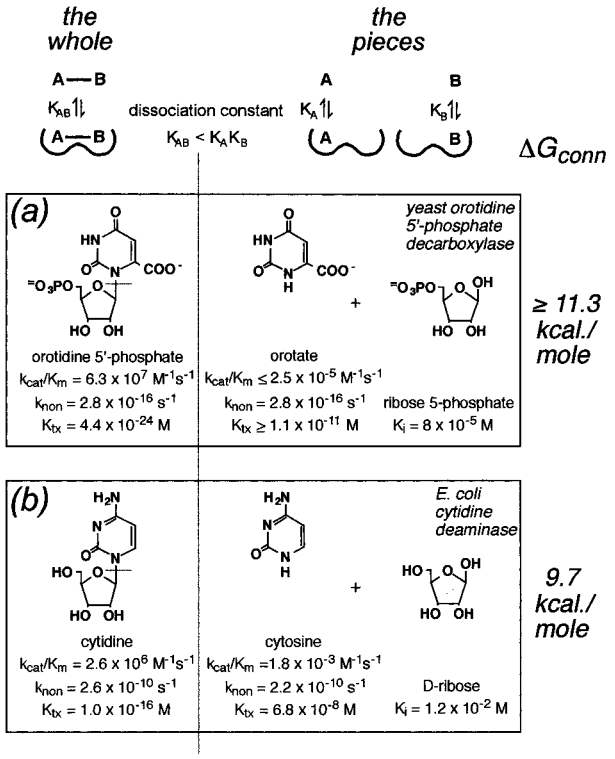

FIGURE 3: Effect of cutting the substrate into two pieces, on enzyme binding affinity for the altered substrate in the transition state, for (a) yeast ODCase (this work) and (b) cytidine deaminase (*21*). In the generic substrate A−B, shown above as "the whole", part A can enhance the effective concentration of part B by reducing its relative freedom of motion. $\Delta G_{conn} = -RT \ln(K_{AB}/K_A K_B)$. This advantage is lost when A and B are disconnected. (The concentration-independent "intrinsic binding free energy" of ribose 5-phosphate in the transition state, obtained by comparing the transition state binding affinity of orotate with that of OMP, is more than 16.6 kcal/mol.)

intermediate or transition state, nor have we observed significant rates of spontaneous decarboxylation of OMP in experiments designed to test that possibility. More significantly, the crystal structure of the BMP complex with yeast ODCase shows that the bound ligand's phosphoryl group is separated from O-2 of its pyrimidine ring by more than 4.5 Å. This separation implies that the bound substrate's phosphoryl group is unlikely to participate directly in its chemical transformation, but has some more physical origin. Of particular interest is the fact that the 10^{12}-fold difference in affinity observed in the transition state for decarboxylation is much larger than the difference between the ground state affinities of OMP ($K_m = 7.0 \times 10^{-7}$ M) and orotic acid ($K_i = 9.5 \times 10^{-3}$ M). Thus, the strength of the enzyme's attraction for the phosphoribosyl group appears to increase as the reaction proceeds from the ES complex through the transition state.

The contribution of the phosphoribosyl group can be analyzed by considering the transition state for enzymatic decarboxylation of OMP "in pieces", as described previously for adenosine (*20*) and cytidine (*21*) deaminases. The apparent dissociation constants (K_{tx}) of orotate and OMP in the transition states for their enzymatic decarboxylation can be obtained in each case by dividing k_{cat}/K_m into k_{non} for the decarboxylation of the model compound 1-methylorotate, at 25 °C (2.8×10^{-16} s^{-1}) (*3*) (Figure 3). If the free energy of ribose 5′-phosphate binding (−5.6 kcal/mol) is added to the apparent free energy of binding of the transition state for decarboxylation of orotic acid (−14.9 kcal/mol or a less negative value), the sum of the binding free energies of these "pieces" of OMP amounts to −20.5 kcal/mol (or a less negative value). In contrast, the apparent free energy of binding of the transition state for OMP decarboxylation is −31.8 kcal/mol. Thus, a substantial portion (>11 kcal/mol in free energy of connection, or an effective concentration of at least 10^8 M) of the substrate's binding affinity in the transition state can be considered to arise from the presence of the covalent linkage between orotic acid and ribose 5′-phosphate. This connectivity effect between functional groups of OMP is near the upper limit estimated as likely to result from restrictions that might be placed on the translational and rotational motions of two binding determinants (A and B) by joining them together in a single molecule (A−B) (*22*). The attainment of such high effective local concentrations is likely to depend on the absence of residual motions in A−B in its unbound form (*22*). Thus, it is of interest that nucleotides show little freedom of rotation in solution, and that ODCase binds nucleotides (*5*−*8*) in a configuration similar to that which is favored by OMP in solution (*23*, *24*).

Recent structural observations are compatible with catalytic mechanisms in which the ground-state enzyme complex with OMP is destabilized relative to the transition state by steric and/or electronic repulsive interactions involving the substrate's 6-carboxylate group (*5*−*8*). The present experiments provide a measure of the importance of the phosphoribosyl group as a source of this differential binding affinity, and of the overall binding affinity that is generated in the transition state for decarboxylation. Its effect is so great that truncation of the substrate at the glycosidic bond produces a "substrate" (orotate) that is bound by the enzyme as a weak inhibitor, but exhibits no detectable activity.

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