CATALYTIC PROFICIENCY: The Unusual Case of OMP Decarboxylase

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Abstract Enzymes are called upon to differ greatly in the difficulty of the tasks that they perform. The catalytic proficiency of an enzyme can be evaluated by comparing the second-order rate constant \( k_{\text{cat}}/K_m \) with the rate of the spontaneous reaction in neutral solution in the absence of a catalyst. The proficiencies of enzymes, measured in this way, are matched by their affinity constants for the altered substrate in the transition state. These values vary from approximately \( \times 10^9 \text{ M}^{-1} \) for carbonic anhydrase to \( \times 10^{23} \text{ M}^{-1} \) for yeast orotidine 5'-phosphate decarboxylase (ODCase). ODCase turns its substrate over with a half-time of 18 ms, in a reaction that proceeds in its absence with a half-time of 78 million years in neutral solution. ODCase differs from other decarboxylases in that its catalytic activity does not depend on the presence of metals or other cofactors, or on the formation of a covalent bond to the substrate. Several mechanisms of transition state stabilization are considered in terms of ODCase crystal structures observed in the presence and absence of bound analogs of the substrate, transition state, and product. Very large connectivity effects are indicated by the results of experiments testing how transition state stabilization is affected by the truncation of binding determinants of the substrate and the active site.

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EVALUATING THE CATALYTIC POWER OF AN ENZYME

In 1923, Wilstätter & Pollinger showed that peroxidase decomposes 1000 times its weight of H₂O₂ per second at 20°C (1). It had already been known that enzymes possess a remarkable talent for easing the difficult transformation of biological molecules from one metastable state to another. This catalytic power, seldom approached by artificial catalysts, can be described in various ways. A typical enzyme-substrate complex forms products with a half-time of 10⁻² to 10⁻³ s under conditions in which the spontaneous reaction, proceeding uncatalyzed, is very slow indeed. During that moment, the strength of the enzyme’s “grip” on the substrate increases by an enormous factor, as shown below.

The decomposition of an enzyme-substrate complex (ES) can be described by a first-order rate constant, the turnover number:

\[ \text{TURNOVER NUMBER: } k_{\text{cat}}(s^{-1}) \]

\[ \text{ES} \rightarrow \text{E} + \text{P} \]

\[ \frac{d[P]}{dt} = (k_{\text{cat}}) [\text{ES}] \]

where E is the enzyme, S the substrate, and P the product.

Under physiological conditions, the concentration of the substrate is usually subsaturating, which allows the rate of the enzyme reaction to respond to changing substrate concentrations. The rate of product formation is then described by the second-order rate constant \((k_{\text{cat}}/K_m)\), termed the catalytic efficiency:

\[ \text{CATALYTIC EFFICIENCY: } k_{\text{cat}}/K_m \text{ (M}^{-1}\text{s}^{-1}) \]

\[ \text{E} + \text{S} \rightarrow \text{E} + \text{P} \]

\[ \frac{d[P]}{dt} = (k_{\text{cat}}/K_m) [\text{E}][\text{S}] \]

When the catalytic efficiencies of a group of enzymes, arbitrarily chosen from the literature, are displayed on a vertical scale (Figure 1), their values lie in the range from 10⁵ to 10⁹ M⁻¹ s⁻¹, clustering around a mean value of \(\sim 10^7\) M⁻¹ s⁻¹ that
does not fall far short of the maximum rate permitted by the frequency of encounter of two molecules of similar radius in water at room temperature. For those enzymes shown in green, the response of the reaction rate to changing viscosity indicates that \( \frac{k_{\text{cat}}}{K_m} \) is at the limit imposed by the rate of enzyme-substrate encounter in solution (for details, see the Appendix).

It has long been recognized that biological reactions vary in their spontaneous rates in neutral solution, so that efficient enzymes differ considerably in the severity of the tasks that they perform. The hydration of \( \text{CO}_2 \), for example, occurs spontaneously within a matter of seconds in neutral solution, whereas the phosphodiester bonds of DNA must be able to withstand spontaneous hydrolysis for long periods of time in the absence of a nuclease if DNA is to serve its purpose in conserving genetic information. Thus, although enzymes are similar

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**Figure 1** Catalytic efficiencies \( (k_{\text{cat}}/K_m) \) reported for enzymes of various types. For enzymes shown in green, \( k_{\text{cat}}/K_m \) has been shown to be diffusion-limited. For enzymes shown in blue, \( k_{\text{cat}}/K_m \) appears to be partly diffusion-limited. For enzymes shown in red, \( k_{\text{cat}}/K_m \) is not diffusion-limited. For enzymes shown in black, the relationship between \( k_{\text{cat}}/K_m \) and diffusion does not appear to have been established. For details, see Appendix.
in their efficiency (Figure 1), they differ in the severity of the tasks that they are called upon to perform. To obtain a quantitative measure of the degree of difficulty of that task for any enzyme, it would be desirable to know the rate constant \( k_{\text{non}} \) of the corresponding reaction proceeding spontaneously in dilute aqueous solution in the absence of a catalyst.

**UNCATALYZED RATE CONSTANT: \( k_{\text{non}} \, (s^{-1}) \)**

\[
S \rightarrow P
\]

\[
d[P]/dt = (k_{\text{non}})[S]
\]

By comparing the rate constant of an uncatalyzed reaction with the turnover number of the corresponding enzyme reaction, it would be possible to appreciate the increase in reaction rate that an enzyme produces. The resulting rate enhancement (the dimensionless ratio of two first-order rate constants) indicates the factor by which an enzyme’s equilibrium affinity for the substrate increases in passing from the ground state to the transition state, as discussed later.

**RATE ENHANCEMENT**

\[
k_{\text{cat}}/k_{\text{non}}
\]

Under the conditions prevailing in vivo, an enzyme is usually not saturated with substrate (2), and its catalytic effect can be measured by dividing the catalytic efficiency (a second-order rate constant) by the first-order rate constant for the uncatalyzed reaction under similar conditions. The resulting expression, which we term “catalytic proficiency” (3), has the dimensions of \( M^{-1} \).

**CATALYTIC PROFICIENCY**

\[
(k_{\text{cat}}/K_m)/k_{\text{non}} \, (M^{-1})
\]

In addition to providing another measure of the catalytic effect of an enzyme, different from the rate enhancement, catalytic proficiency provides a measure of the affinity that an enzyme develops for the substrate as they pass through the transition state, and the corresponding susceptibility of an enzyme to inhibition by a stable compound that resembles the altered substrate in the transition state.

**ENZYME-SUBSTRATE BINDING AFFINITY IN THE GROUND STATE AND TRANSITION STATE**

“It can be seen that the activation energy of the catalyzed reaction is lowered on the surface to a smaller value and that this decrease, apart from the corrections due to the free energy of adsorption of the substrate in the ground state, is principally caused by the large negative free energy of adsorption of the activated state. The resulting concept of the action of a catalyst may thus be expressed: the
energy barrier to be overcome is lowered in the adsorption layer because the activated state is strongly adsorbed and therefore, in the adsorption layer, is formed in a less endergonic process and is therefore more often reached. Hence, it is not that the adsorbate is activated but that the adsorbate is more easily activated and is therefore, at equilibrium, present in the activated state to a greater percentage than in the free state.”

These words, written by M. Polanyi 80 years ago [(4), see also (5)], describe the action of a catalyst in thermodynamic terms that are general for any reaction that does not involve quantum mechanical tunneling of protons. Polanyi’s formalism, shown in Figure 2, does not depend on the nature of the reaction or the solvent environment (in fact it was originally developed to describe vapor phase reactions at catalytic surfaces) nor does it require that the catalyst be flexible or rigid. Those are some of the details that constitute the mechanism in a particular case and can be determined by experiment.

Figure 2 Polanyi’s view of the relationship between free energy of activation for spontaneous and catalyzed reactions and free energies of binding of the reactant in the ground state and the transition state (4, 5). Note that the rate enhancement is matched by the enhancement in binding affinity as the substrate proceeds from the ground state (“adsorbed reactant”) to the transition state (“activated adsorbed reactant”).
A conceptual advantage of transition state theory is that it focuses attention on a structure rather than a process. Using the conventional experimental techniques available to enzymologists and physical organic chemists (kinetic measurements, substituent and isotope effects, and linear free energy relationships), and the assumption that the ground state and the transition state are in equilibrium, it is possible to characterize the transition state of many reactions in considerable detail (5a).

During the past 32 years, Polanyi’s relationship has provided a basis for the design of enzyme inhibitors, termed transition state analogs, that find the Achilles’ heel of these proficient catalysts [for recent reviews, see (6–9)]. These inhibitors take advantage of the implied increase in binding affinity as a bound substrate proceeds from the ground state to the transition state (Figure 2), which matches and may exceed (10) the rate enhancement that an enzyme produces. It should be added that this relationship does not apply, in its simplest form, to enzyme reactions that involve quantum mechanical tunneling of protons, and that it requires modification for reactions proceeding through intermediates that involve the transient formation of a covalent bond between the enzyme and the substrate (10, 11). The crystal structures of enzyme complexes with transition state analogs have been used to identify and explore the function of active site residues that are involved in substrate transformation. These structural studies have also uncovered a general tendency of enzyme active sites to envelop the altered substrate (Figure 3), allowing the number and strength of binding interactions to be maximized in the transition state (12) in a way that would not have been anticipated if the free energies of forming these bonds were additive (13). The distinction that was once made between “binding” and “catalytic” groups at the active sites of enzymes now appears somewhat artificial, because catalysis requires that binding affinity approach a maximum in the transition state (10).

If one accepts the equilibrium assumption on which transition state theory is based, one must also accept the limitations implied by that assumption. In most cases, the ground state and the transition state are the only species whose thermodynamic and structural properties are open to experimental verification by structural and kinetic methods (14). Pathways from the ground state to the transition state and from the transition state to the product are not specified, and multiple pathways may be available. For steric reasons, these pathways are likely to be more restricted for enzyme-catalyzed reactions than for the corresponding nonenzymatic reactions, but the structures and properties of high-energy intermediates that might lie along any particular path to and from the transition state tend to be inaccessible to experimental investigation, simply because the population of these intermediates becomes infinitesimally rare as they approach the transition state in structure and free energy (15).

One path to the transition state can be ruled out, however, for most enzyme reactions. An enzyme could not produce much catalysis by “fishing” for highly activated forms of the substrate that are present in solution, i.e., forms of the substrate that approach the transition state in structure and free energy, because
they are too rare. Even in the fastest known enzyme reactions, such as that catalyzed by carbonic anhydrase for which $k_{\text{cat}} = 10^6 \text{ s}^{-1}$ (16), the value of the equilibrium constant ($K^\ddagger$) is $10^6/(kT/h) \approx 10^{-7}$, where $k$ is Boltzmann’s constant and $h$ is Planck’s constant. Thus, the equilibrium constant ($K^\ddagger$) for conversion of ES to ES$^\ddagger$ is always exceedingly unfavorable. Most enzyme reactions proceed with efficiencies that approach within about two orders of magnitude the limit

![Figure 3](image-url)
We are lead to infer that an efficient enzyme reaction (with a high value of $k_{\text{cat}}/K_m$) must proceed by combination of E with S in forms that are not very rare, or chemically activated, in solution (12). That sequence of events does not preclude the possibility that in the ES complex in the ground state, the bound substrate experiences a destabilizing effect near its scissile bonds that is “paid for” by strong binding interactions involving parts of the substrate that are distant from the site of bond cleavage. Noting that distant binding determinants of a substrate are often found to influence $k_{\text{cat}}$ more strongly than they influence $K_m$, Jencks has compared their attractive influence to that of Circe and her ladies before she transformed Odysseus’s men into swine: “This anchoring effect provides a rationale for the large size of enzymes, coenzymes and other substrates. Energy from the specific binding interactions between an enzyme and a substrate or coenzyme is required to bring about the highly improbable positioning of reacting groups in the optimum manner and such binding requires both a high degree of three-dimensional structure and a large reaction area” (17). Thus, ground state destabilization in some form is likely to be present in the reactive portion of the substrate in the ES complex, even though the complex as a whole is thermodynamically stable. It should be noted that the introduction of such an effect is expected to elevate $k_{\text{cat}}$. Because it elevates $K_m$ to the same extent, the magnitude of $k_{\text{cat}}/K_m$ is unaffected.

THE RATE ENHANCEMENTS PRODUCED BY ACTUAL ENZYMES

Until recently, the rates of many biological reactions had been considered to be too slow to measure. If reaction mixtures are introduced into sealed quartz tubes (typically 3 mm in diameter; 1 mm wall thickness) at elevated temperatures, it is possible to follow reactions in neutral aqueous solution at temperatures up to 374°C, the critical point of water. The vapor pressure of water causes these tubes to explode at temperature above 260°C, but explosions can be avoided by enclosing tubes in steel bombs, along with additional water to equalize the pressure across the walls of the reaction vessels. At high pH values, and at high concentrations of anionic buffers, it is advisable to use Teflon (PTFE) vessels enclosed in steel bombs, because silicate is etched from the walls of the tubing under these conditions. If, as is usually the case, rate constants obtained in this way yield linear Arrhenius plots, these can be used to obtain rate constants at ambient temperatures and establish benchmarks for comparison with reactions catalyzed by enzymes (Figure 4).
Which enzymes produce the largest rate enhancements? The rate constants of biological reactions proceeding spontaneously in the absence of a catalyst (Figure 5) span a range of at least 15 orders of magnitude, ranging from $2.8 \times 10^{-2} \text{ s}^{-1}$ for the hydration of CO$_2$ to $2 \times 10^{-17} \text{ s}^{-1}$ for the decarboxylation of glycine in neutral solution at 25°C. In contrast, most enzyme reactions proceed at rates that fall within a relatively narrow range (Figure 1), as is necessary if they are to be useful at the limited concentrations at which they are present in the cell. Because of this difference in range, the rate enhancements that enzymes produce tend to reflect differences in the rates of the corresponding nonenzymatic reactions. At one extreme, chorismate mutase produces a rate enhancement of only $\sim 10^6$ (3), whereas several major classes of enzymes are found to enhance reaction rates by factors of more than $10^{15}$. These include amino acid decarboxylases, glycosidases, phosphodiesterases and phosphomonoesterases, and OMP decarboxylase (18). It is of interest to consider each of these extreme cases in terms of the probable mechanism of action of the enzyme.

Amino acid decarboxylases enhance reaction rates by a factor of almost $10^{20}$ (19, 20). So far as is known, every one of these enzymes acts with enzyme-bound pyridoxal or pyruvoyl groups as cofactors, so that there is a fundamental difference in mechanism between the enzymatic and uncatalyzed reactions. For
these reactions, the transient formation of a covalent bond between the substrate and the enzyme-bound cofactor supplies a route for delocalization of negative charge in the transition state for decarboxylation.

Glycosidases can be divided into two categories, acting by mechanisms that may involve either (a) hydrolysis of a glycosyl-enzyme intermediate, with two inversions, leading to overall retention of configuration at the carbon atom where substitution occurs, or (b) direct water attack, with a single inversion of configuration at the scissile carbon atom. Sweet potato β-amylase, an enzyme of the latter type, enhances the rate of glycoside
hydrolysis by a factor of $\sim 10^{17}$ (21). Reactions of this type are subject to acid catalysis, for which most glycosidases supply appropriate carboxylic acid residues at their active sites (22).

The enzymatic hydrolysis of phosphate monoesters often proceeds by formation of an intermediate with a phosphorylated serine residue. That is the case for bacterial alkaline phosphatase, which contains zinc in a position to activate the serine for nucleophilic attack (23). In the absence of enzyme, the rate of monoester hydrolysis is at least $10^{15}$-fold slower (24), and recent evidence indicates that the actual rate of spontaneous hydrolysis of phosphate monoester dianions is slower still (N. H. Williams & R. Wolfenden, unpublished data).

The spontaneous rate of phosphodiester hydrolysis is also very slow, as indicated by the stability of the bonds in DNA. In neutral solution, hydrolysis of dimethyl phosphate exhibits an apparent $k_{\text{non}}$ of $10^{-13}$ s$^{-1}$, but hydrolysis occurs almost entirely by C–O cleavage, implying that P–O cleavage must be roughly two orders of magnitude slower. The rate constant estimated for this latter process ($10^{-15}$ s$^{-1}$, compared with the turnover number of staphylococcal nuclease, indicates that this metalloenzyme produces a rate enhancement of $\sim 10^{17}$-fold (24).

OMP decarboxylase, which also produces a very large rate enhancement ($\sim 10^{17}$-fold), is unusual among these enzymes in that it acts without metals or other cofactors, by a mechanism for which there seems to be no obvious chemical precedent. Before discussing the properties of this enzyme, it is of interest to consider some possible sources of the catalytic proficiencies of enzymes in general (Figure 6), and their corresponding affinities for the altered substrate in the transition state.

WHAT IS THE SOURCE OF TRANSITION STATE AFFINITY?

That question can be addressed in thermodynamic or structural terms. Although the answer varies from case to case, several common tendencies have been noted. Many uncatalyzed reactions are much more temperature-sensitive than would be expected on the basis of a traditional rule of thumb, which states that aqueous reaction rates tend to double ($Q_{10} = 2$) with a $10^\circ$C rise in temperature. A recent survey has uncovered $Q_{10}$ values as large as 6 for glycoside hydrolysis, and even larger values for the decarboxylation of aromatic acids. In contrast, enzyme reactions are relatively insensitive to temperature, with typical $Q_{10}$ values of 1.5–2.0. As a result, enzyme rate enhancements increase sharply with decreasing temperature, and the sensitivities of enzymes to transition state analog inhibitors undergo a corresponding increase as temperature is lowered, with only one exception of which we are aware (24a). These results indicate that enzymes are distinguished from many artificial catalysts by their ability to reduce a reaction’s
Figure 6  Values of $k_{\text{non}}$ and $k_{\text{cat}}/K_m$ for enzymes that do not appear to act by forming covalent intermediates, including arginine decarboxylase (ADC), ODCase (ODC), sweet potato β-amylase (GLU), fumarase (FUM), mandelate racemase (MAN), carboxypeptidase B (PEP), cytidine deaminase (CDA), ketosteroid isomerase (KSI), chorismate mutase (CMU), and carbonic anhydrase (CAN). The length of each vertical bar represents the enzyme’s catalytic proficiency or minimal affinity for the altered substrate in the transition state. [For references, see (18)].
heat of activation, a tendency that may reflect selective pressures that were at work during the early stages of evolution on a cooling Earth (25).

To produce these large rate enhancements, very large increases in affinity must occur as an enzyme-substrate complex progresses toward the transition state, greatly surpassing the enzyme’s affinity for the substrate in the ground state (Figure 2). The magnitudes of these increases in affinity are especially impressive in that they are usually achieved without the formation of covalent bonds between the catalyst and the substrate. The sharp temperature sensitivity of transition state binding affinity would be understandable if certain H-bonding and electrostatic interactions were present between the enzyme and substrate in the transition state that were not present in the ground state enzyme-substrate complex. Formation of polar bonds of these types is typically associated with a release of enthalpy. In the case of cytidine deaminase, for example, the contribution of a single H-bonding interaction (involving the 2′-OH group of the substrate) has been analyzed, either by removing that group or by deleting the H-bonding residue at the active site. Either of these operations results in a major loss of transition state binding affinity, and that loss is due to a reduction of the heat released upon transition state binding (26).

The contributions of individual binding determinants to transition state affinity can be analyzed in another way by comparing the rate constant of the uncatalyzed reaction, first with the rate constant observed for the native enzyme acting on its natural substrate, and then with the rate constants observed for each of two “pieces” obtained by truncating the enzyme’s active site (a mutant enzyme and a molecule representing its missing side-chain). In that way, the transition state affinity of the native enzyme can be compared with those of two pieces produced by a binary “cut” that leaves these pieces intact in other respects. The benefit to catalysis that an enzyme derives from having the two parts properly connected has been found to amount to as much as ~10^8 M in several cases (18). These observations give some quantitative sense of the magnitude of the benefits that arise from the presence of a discrete three-dimensional structure in enzyme active sites.

HOW SLOW ARE DECARBOXYLATION REACTIONS?

In catalyzing decarboxylation reactions, enzymes face the challenge of stabilizing carbon anions formed by elimination of CO_2. With certain well-known exceptions such as nitromethane and thiamine, carbonions are formed with extreme difficulty in water. Thus, acetic and benzoic acids survive exposure for several weeks to neutral aqueous solution at 360°C (just below the critical point of water) without decarboxylation, and neither methane nor benzene undergo appreciable deuterium exchange with solvent water under the same conditions (R. Wolfenden, unpublished data). When a nitrogen atom with a partial positive charge is present next to the scissile carbon atom, decarboxylation becomes
appreciably less difficult, so that it can be measured. In the absence of a catalyst, the half-time for spontaneous decarboxylation of orotidine 5'-phosphate is only (!) 78 million years (3), and the half-time for decarboxylation of glycine is 1.1 billion years (19).

One mechanism by which enzymes might catalyze reactions in which electrostatic charge is reduced or delocalized in the transition state, relative to the ground state, is “catalysis by desolvation” (27). In model studies of the enzymatic decarboxylation of pyruvate, Lienhard & associates observed a major increase in the rate of decarboxylation in dioxan and alcohols compared with water (28, 29). Kemp & Paul observed a $\sim 10^8$-fold increase in the first-order rate constant for decarboxylation of benzisoxazole-3-carboxylic acids when these reactions were carried out in the dipolar aprotic solvent hexamethylphosphoramide (30), and effects almost as large have been observed for the hydrolysis of $p$-nitrophenyl phosphate (31). Desolvation may also explain the cyclodextrin-mediated catalysis of phencylcymoacetate and benzylyacetate decarboxylations (32, 33, 34). That desolvation effects might play a role in enzymatic decarboxylations is indicated by $^{13}$C kinetic isotope effects on arginine decarboxylase in which decarboxylation appears to become less rate determining in the presence of increasing concentrations of ethylene glycol (35). The hypothesis that enzymes use desolvation as a catalytic strategy in decarboxylation reactions has been questioned because desolvation in the ground state would presumably exact a heavy penalty in free energy (36). Thus, desolvation might be expected to offer one means of increasing $k_{\text{cat}}$ (and $K_m$), but would not be expected to enhance catalytic efficiency ($k_{\text{cat}}/K_m$).

**STRATEGIES FOR ENZYMATIC DECARBOXYLATION**

Most decarboxylases use cofactors to delocalize the negative charge that is generated by elimination of CO$_2$ from the substrate. In many $\alpha$-amino acid decarboxylases, the aldehydic group of pyridoxal phosphate, initially bound by a lysine residue as an aldimine, undergoes rapid transimination to the $\alpha$-amino group of the substrate; alternatively, $\alpha$-amino acids are sometimes decarboxylated by condensation of the amino group with a pyruvoyl cofactor, whose carboxyl group remains covalently attached through an ester linkage to the enzyme. Formation of the aldimine or ketimine delocalizes the negative charge that is generated by elimination of CO$_2$; and even such a weak electrophile as acetone has recently been shown to catalyze the racemization of amino acids (37). Thiamine pyrophosphate is the usual cofactor for decarboxylases that act on $\alpha$-keto acids such as pyruvate and isocitrate. In these reactions, ionization of the proton at the 2-position of the thiamine ring results in formation of an anion that condenses with the $\alpha$-keto group of the substrate, introducing a path for delocalization of negative charge.
Metal-dependent decarboxylases, such as oxaloacetate decarboxylase (38), act on \(\beta\)-keto acid substrates by using a divalent cation as a repository for delocalizing negative charge. In another group of enzymes acting on \(\beta\)-keto acids such as acetoacetate, the keto group of the substrate condenses with a lysine residue to form a ketimine intermediate that undergoes decarboxylation more easily (39). \(\beta\)-Hydroxy acids undergo enzymatic decarboxylation through their initial oxidation to \(\beta\)-keto acids. Oxidative decarboxylases of this type, which include the malic enzyme, prephenate dehydrogenase, and isocitrate dehydrogenase employ NAD or NADP as a cofactor [for a recent review of enzymatic oxidative decarboxylations, see (40)].

In each of the reactions mentioned above, the cofactor is an effective catalyst by itself, so that the catalytic burden borne by the protein is relatively modest. Thus, \(\text{CO}_2\) is released from oxaloacetate by divalent cations (41, 42, 43), pyruvate is decarboxylated by thiamine pyrophosphate (44), and alanine is decarboxylated in the presence of pyridoxal phosphate (20). PLP alone has been shown to enhance the rate of arginine decarboxylation by a factor of \(2 \times 10^{11}\), leaving the protein to contribute a relatively modest factor of \(\sim 10^8\) to the reaction rate (19, 20).

OMP decarboxylase differs from all the enzymes mentioned above in that its catalytic activity does not depend on the presence of metals or other cofactors, or on the formation of a covalent bond to the substrate. Instead, this enzyme appears to act as a pure protein catalyst, catalyzing the reaction shown in Figure 7 through noncovalent binding interactions that involve only the functional groups of its constituent amino acids (45, 46, 47).

**SUBUNIT EQUILIBRIA AND CATALYTIC BEHAVIOR OF OMP DECARBOXYLASE**

In yeast and bacteria, ODCase occurs as a protein that appears to have a single function. In mammals, the enzyme that catalyzes OMP decarboxylation constitutes part of a bifunctional enzyme named UMP synthase that also catalyzes the preceding reaction in pyrimidine nucleotide biosynthesis, the transfer of ribose 5-phosphate from 5-phosphoribosyl-1-pyrophosphate to orotate to form OMP (48). Two inherited disorders affecting pyrimidine biosynthesis are the result of deficiencies in the bifunctional enzyme. Both disorders can be treated with uridine or cytidine, either of which lead to increased UMP production via the action of nucleoside kinases. The resulting UMP inhibits carbamoyl phosphate synthase, attenuating orotic acid production.

ODCase from *Saccharomyces cerevisiae* has been studied intensively with respect to its kinetic properties (49, 50, 51, 52) and the physical behavior of its subunits (48, 52, 53). Porter & Short (52) recently used steady-state and pre-steady-state kinetic procedures, together with the intrinsic fluorescence of yeast ODCase, to characterize the enzyme’s state of subunit aggregation, the
The reaction catalyzed by ODCase, showing the affinities of the yeast enzyme for its substrate, the product and two competitive inhibitors: 6-hydroxyUMP (or BMP) and 6-azaUMP. 6-HydroxyUMP bears some resemblance to the carbanion shown in brackets (58). RP represents a 5’-phosphoribosyl substituent.

relation between aggregation and enzyme activity, the stoichiometry of substrate binding, the possibility of interaction between substrate binding sites, and the rates and sequence of events associated with substrate turnover. The dimeric (E₂), but not the monomeric (E), form of the enzyme was shown to be active (52). Dimerization is promoted by substrate binding (48) or by addition of NaCl. The inactivity of the monomer is explained by the crystal structure described later, which shows that each of the active sites (two per dimer) is situated at the interface between identical subunits, and that both subunits contribute side chains that are essential to catalysis. The activity of highly diluted enzyme, largely monomeric, was found to increase with time in a substrate-dependent manner, as expected if substrate were bound only by the dimeric enzyme. The equilibrium constant for subunit dimerization was $2.3 \times 10^7 \text{ M}^{-1}$ under the conditions of the experiment, and the rate of dimerization was found to be consistent with “trapping” of ligands by the active dimer (E₂) after its formation from the monomer in a ligand-independent second-order process with a rate constant of $1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (53).
The binding of OMP by E₂ is accompanied by quenching of the intrinsic fluorescence of the protein that allows the rate of substrate binding to be monitored. Using NaCl to raise the dissociation constant of E₂-OMP complex, Porter & Short were able to measure the rate constant for substrate binding, and to monitor the release of UMP by the return of fluorescence (52). The rate constant for a single turnover, obtained in this way, agreed with the value of \( k_{\text{cat}} \) measured conventionally, and was consistent with the view that the two active sites operate independently. The substrate and other ligands were found to be released at rates that varied in rough proportion to their equilibrium binding affinities, whereas the rate constants for the uptake of various nucleotides were approximately identical. Values of \( k_{\text{cat}} / K_m \) for OMP were found to reach an optimum at a pH of \(~6.9\) (54). Successful enzyme-substrate encounter appears to require the conjugate base of a group with a pKₐ of 6.1 and the conjugate acid of a group with a pKₐ of 7.7 in 0.1 M NaCl. The higher pKₐ value was tentatively ascribed to a group on the enzyme, possibly Lys-93 (see below), whereas the lower pKₐ value was ascribed, at least in part, to the substrate phosphoryl group. The pH dependence of \( k_{\text{cat}} \) was also bell shaped, with pKₐ values (4.7 and 8.8) somewhat more widely separated than those observed for \( k_{\text{cat}} / K_m \) (51). By conducting single-turnover experiments using a fluorescein dye with a pKₐ value of \(~7\), in the presence and absence of carbonic anhydrase, Porter & Short also showed that the immediate product of substrate decarboxylation is CO₂ rather than bicarbonate (52).

The value of \( k_{\text{cat}} / K_m \) for ODCase does not change with major increases in solvent viscosity (55) but shows a \(^{13}\)C isotope effect at the scissile C-C bond (54) that is considerably larger than the \(^{13}\)C isotope effect observed for the uncatalyzed reaction (56). The absence of a viscosity effect on \( k_{\text{cat}} / K_m \) indicates that \( k_{\text{cat}} / K_m \) is not diffusion-limited, and the absence of a viscosity effect on \( k_{\text{cat}} \) indicates that \( k_{\text{cat}} \) is not limited by product release (55). These results accord with the view that \( K_m \) represents a true dissociation constant for the enzyme-substrate complex, and that \( k_{\text{cat}} / K_m \) describes the chemical decarboxylation of the substrate. The nature of the enzyme group with an apparent pKₐ value near 7.7 remains to be established. Although this ionization might be associated with Lys-93, the location of that residue adjacent to two carboxylate groups in the active site (see discussion of structure below) would ordinarily be expected to elevate its pKₐ above the values of \(~10.5\) that are typical of the side-chains of peptide lysine residues in contact with solvent water.

**POSSIBLE MECHANISMS OF ENZYMATIC DECARBOXYLATION OF OROTIDINE 5’-PHOSPHATE**

In a first attempt to elucidate the mechanism of this reaction, Beak & Siegel (57, 58) showed that 2-methylation of 1-methylorotate acid led to an increase of more than \(10^8\)-fold in the rate of spontaneous decarboxylation. They proposed that an
acidic group on the enzyme might perform a similar function by protonating O-2 of the substrate, to generate a zwitterionic form of OMP with a proton attached to N-3. Decarboxylation of 1,3-dimethylorotic acid at elevated temperatures in sulfonamide proceeded relatively easily, consistent with the possibility that decarboxylation is initiated by formation of the 1,3-dimethylorotate zwitterion as shown in Figure 8a.

Westheimer & associates (59) observed that 6-hydroxyUMP (the 5'-phosphoribosyl derivative of barbituric acid, which we will term "BMP") was a potent competitive inhibitor of yeast ODCase, with $K_i = 8.8 \times 10^{-12}$ M and noted its structural resemblance to the carbanionic intermediate generated by decarboxylation (Figure 7). That value may be compared with $K_m = 7 \times 10^{-7}$ M for OMP, a value that appears to represent a true dissociation constant for enzyme and substrate (55). These investigators noted the apparent preference of the enzyme for binding anionic ligands, and suggested the possible presence of positively charged groups at the active site. A search for positively charged residues of the yeast enzyme that might protonate the substrate during decarboxylation led to the identification by Smiley & Jones of Lys-93 as essential for catalysis (60). Mutation of that residue reduced enzyme activity by a factor of more than $10^7$; and its replacement by cysteine, followed by reaction with 2-bromoethylamine, led to partial recovery of activity. These authors proposed that Lys-93 might serve as the Brønsted acid that protonates O-2 of OMP according to the Beak & Siegel mechanism (Figure 8a).

Silverman & Groziak proposed an alternative mechanism for decarboxylation that would involve formation of a covalently bound intermediate (61). By analogy to the reaction catalyzed by thymidylate synthase, these authors suggested that enzymatic decarboxylation might proceed through addition of an active site nucleophile at the 5=6 double bond of OMP (Figure 8b). The resulting change in bond hybridization, if that had proceeded to any considerable extent during the rate-determining step, would be expected to lead to a secondary kinetic isotope effect on $k_{cat}/K_m$ if deuterium were substituted at C-5, and to a change in electron density at C-5 if the potential transition state analog 6-hydroxyUMP were similarly bound. When these possibilities were tested (the latter effect by $^{13}$C NMR, using 6-hydroxyUMP labeled with $^{13}$C at the 5-position), neither prediction was fulfilled. Thus, C-5 does not seem to undergo a detectable change in geometry during the substrate’s progress from the ground state to the transition state for enzymatic decarboxylation (62). Later, that conclusion was placed on a firmer footing when the rate-limiting step for $k_{cat}/K_m$ was shown, by $^{13}$C and solvent deuterium isotope effects, to involve a proton-sensitive step with partial cleavage of the C–C bond of the substrate (54, 63).

Citrazinic acid, the 1-deaza analog of 1,3-dimethylorotic acid, was found by Wu et al. to undergo spontaneous decarboxylation at a rate comparable with that observed for 1,3-dimethylorotic acid (64). These investigators pointed out that the decarboxylation of citrazinic acid should have been many orders of magnitude slower than that of 1,3-dimethylorotate if the zwitterion mechanism had been operative in the spontaneous reaction, since citrazinic acid is incapable of forming a similar zwitterion. $^{13}$C kinetic isotope effects on the decarboxylation
of 1,3-dimethylorotate also appear to support a mechanism that does not involve formation of a ylide at N-1 in the uncatalyzed reaction (65). Rishavy & Cleland (66)
measured kinetic isotope effects of nitrogen on $k_{cat}/K_m$ in the enzyme-catalyzed reaction, and found no evidence of a change of bond order at N-1 during the rate-determining step for $k_{cat}/K_m$. Since the rate-limiting step had been shown to involve C–C cleavage (54), these authors inferred that the $^{15}$N isotope effects ruled out formation of a nitrogen ylide before or during C–C bond cleavage in the enzyme reaction.

Gas-phase proton affinities, calculated for orotate by Lee & Houk, suggest that O-4 rather than O-2 is the most likely site of protonation of OMP in the vapor phase (67), and simulations of the reaction in a nonpolar environment suggest that the spontaneous decarboxylation of orotate may proceed by a mechanism that involves protonation at O-4, followed by formation of a neutral carbene at C-6 (Figure 8c). Moreover, the enthalpy of activation estimated by these investigators for decarboxylation of 4-protonated orotate proved to be comparable with the enthalpy of activation determined later for the enzyme reaction (68). Notwithstanding that similarity, the crystal structures of OMP decarboxylase (discussed below) indicate that the active site is relatively polar, instead of providing a low dielectric environment of the kind that would be expected to promote a carbene-based mechanism. Moreover, no acidic residue appears to be present near O-4 of a bound substrate analog, a bound transition state analog, or the reaction product, reducing the likelihood of a simple carbene-based mechanism. A revised version of the carbene-based mechanism has been described recently (69) that would involve protonation at O-4 by a bound water molecule, followed by formation of the neutral carbene intermediate, as in (70).

To what extent is the uptake of a proton concerted with the departure of CO$_2$? Although that question remains to be settled, recent calculations based on the No Barrier Theory seem to be consistent with the view that the transition state has considerable carbanion character (P. Guthrie, personal communication). It also seems clear that the carbanion is an extremely high energy species in water, as the apparent pK$_a$ value of C-6 of UMP has recently been found to be in the neighborhood of 34, comparable with the analogous ionizations of thiophene and indole (A. Sievers & R. Wolfenden, unpublished experiments).

THE STRUCTURE OF OMP DECARBOXYLASE—NEW MECHANISTIC INSIGHTS?

Five years after the discovery of its remarkable catalytic proficiency, ODCase was crystallized almost simultaneously in four laboratories, from *Saccharomyces cerevisiae* (71) and from three species of bacteria (72, 73, 74), in a complex with three different ligands. In most respects, these structures are in very close agreement [for discussion, see (69) and (75)]. ODCase adopts an α/β-barrel fold with a ligand binding site consisting of residues belonging to both subunits of the active homodimer, Figure 9. This Figure summarizes the enzyme-ligand contacts that are observed for the complex formed between the yeast enzyme and the
Figure 9. Schematic view of the active site of yeast ODCase (71), showing the active site residues that contact the inhibitor 6-hydroxyUMP (BMP). Positively charged residues are shown in red, negatively charged residues in green.
transition state analog 6-hydroxyUMP (BMP). Comparison of the free and liganded states of yeast ODCase shows that significant changes in enzyme conformation accompany the binding of the transition state analog BMP (71). A looped region comprising residues 207–217 of the yeast enzyme closes over the active site, interacting with the phosphoryl group of the bound nucleotide and excluding bulk solvent water from the binding cavity.

Numerous favorable H-bonds and electrostatic interactions are present in the BMP complex that involve the phosphoryl group, ribofuranosyl group, and pyrimidine ring. Eight invariant amino acids appear in the enzyme from more than 80 species, and seven of these make direct contact with the substrate (77). Particularly conspicuous is the presence of a novel quartet of alternately charged residues in the active site, including Lys-59, Asp-91, Lys-93, and Asp-96 of the yeast enzyme, in the neighborhood of C-6 where decarboxylation occurs. Also of interest are the enzyme’s extensive interactions with the phosphoryl group, helping to explain the very large contribution that the phosphoryl group makes to catalysis (see below). Surprisingly, the substrate’s carbonyl groups are associated with the side-chain –NH₂ group of Gln-215 and the backbone –NH- group of Ser-154, neither of which seems likely to be effective as a general acid catalyst. The side-chain of Lys-93 is located near C-6 of the substrate, in a position to optimize electrostatic interactions with negative charge developing at C-6 of the substrate during CO₂ elimination. At some point in that process, Lys-93 might be expected to transfer its proton to C-6, generating the uncharged reaction product UMP for which the enzyme exhibits a relatively weak affinity (Figure 7). This general mechanism may provide a partial explanation of the high catalytic proficiency of ODCase, but it leaves one important question unanswered. The rate enhancement (k₉₀/k₉₀) produced by any catalyst depends on its ability to bind the substrate in the ground state less tightly than it binds the altered substrate in the transition state (Figure 2). The remarkable rate enhancement (k₉₀/k₉₀) produced by ODCase implies a corresponding difference in binding affinity between the substrate in the ground state and the transition state. How is such a high level of binding discrimination achieved by such a simple arrangement of residues at the active site?

To answer that question definitively will require detailed knowledge of the configuration of the enzyme-substrate complex in the ground state. Because the reaction is almost irreversible (Kₑq > 10⁶ M)(B. G. Miller & R. Wolfenden, unpublished experiments), and the ES complex species leads such a fleeting existence under physiological conditions, any inferences about its structure must necessarily be indirect, at least for the present. If Lys-93 stabilizes a carbanionic transition state, then it is of special importance that this residue be prevented from interacting equally favorably with the –COO⁻ group of the substrate in the ground state enzyme-substrate complex. Efforts to model OMP into the binding site observed for BMP in the yeast enzyme suggested that the –COO⁻ group group of OMP could not replace the –O⁻ group of BMP without severe steric crowding (71), leading to the conjecture that the network of charged residues is
important in constraining the position of the side-chain of Lys-93 with respect to the bound substrate in the ground state and transition state.

Using the structure that they had determined for the complex formed between the thermophilic enzyme and the product analog 6-AzaUMP, Wu et al. combined quantum mechanics with molecular dynamics calculations in an effort to model OMP into the nucleotide binding site (72). From that simulation, these investigators inferred the existence of repulsive interactions between the substrate carboxylate group and the carboxylate group of the residue corresponding to Asp-91 of the yeast enzyme. They identified this repulsion as a basis for local destabilization of the ES complex, counterbalanced by attractive interactions involving the phosphoribosyl group. Invoking the mythical figure of Circe, these investigators estimated that the phosphoribosyl group of the substrate might supply up to 26 kcal/mol of binding free energy in the ground state ES complex, of which 18 kcal/mol is used to destabilize the reactive portion of the substrate in the ground state.

After determining the structure of the complex formed by the Bacillus subtilis enzyme with product UMP, Appleby et al. suggested a different mechanism that would not involve the formation of an unstable carbanion (Figure 8d) (73). Instead, their mechanism postulates that lysine-93 transfers a proton to C-6 as CO₂ departs, in an electrophilic displacement for which they find some precedent in model reactions (79). Consistent with that possibility, Ehrlich et al. (80) have reported solvent kinetic isotope effects on the ratio of $^{13}$C/$^{12}$C in product CO₂ that they interpret as indicating that proton transfer precedes C–C bond cleavage, although they pointed out the alternative possibility that changes in conformation might be responsible for the effects observed. These authors suggested that decarboxylation might also be promoted by repulsive interactions between the scissile carboxylate group of the substrate and an active site carboxylate group.

Having determined the structure of the Escherichia coli enzyme complex with BMP, the Larsen group suggested an additional mechanistic possibility, based on the apparent proximity of the substrate’s reactive carboxylate group to aspartate-91 (74). In that mechanism, they propose that a short, strong, hydrogen bond is formed between Asp-91 and the reactive carboxyl group of substrate OMP, and that the continued presence of this H-bond in the transition state lowers the activation barrier for decarboxylation. In our view, it would be necessary for that H-bond to become vastly stronger in the transition state than in the ground state for any rate enhancement to result. Larsen & coworkers also postulate that charge repulsion between carboxylate groups of the enzyme and substrate promotes the release of CO₂, as in the ground state destabilization mechanism proposed by Wu et al. (72).

Another mechanistic suggestion was put forward by the late Peter Kollman & associates, who combined molecular dynamics simulations of the thermophilic enzyme’s complex with various ligands with quantum mechanical-free energy calculations of the reaction trajectory (80a). They propose that Lys-93 protonates OMP at C-5 as shown in Figure 8e. However, that proposal seems difficult to reconcile with some of the evidence mentioned earlier. Thus, $^{13}$C kinetic isotope
effects indicate that C-C bond cleavage has progressed to a considerable extent in the transition state (54). If C-5 had undergone rehybridization before CO₂ was released, and that rehybridization were retained in the transition state, then 5-deuteration of OMP might have been expected to result in a substantial secondary isotope effect on \( k_{\text{cat}}/K_m \). That isotope effect is in fact negligible (62). Moreover, the finding that 5-fluoroOMP is almost equivalent to OMP in its \( k_{\text{cat}}/K_m \) value (45) seems difficult to reconcile with rehybridization at C-5 in the transition state for the enzyme reaction as implied by mechanism (e) in Figure 8.

Smiley & associates have suggested yet another possibility (81), based on their interesting finding that 2-thioOMP is at least 10⁷-fold less reactive than OMP as a substrate for yeast ODCase. In addition, 2-thioOMP fails to inhibit the enzyme even at relatively high concentrations, indicating that it is bound at least 10²-fold less tightly than OMP. As the existing crystal structures suggest no obvious reason why 2-thioOMP should be physically prevented from binding in a substrate-like manner, Smiley et al. suggest that these crystal structures might also be misleading with respect to the orientation of substrate OMP. If OMP were bound productively with its pyrimidine ring rotated 180°, placing its carboxylate group near substituent ribose, then Lys-93 could play the role originally assigned to it as a Brønsted acid. A requirement for that mode of binding could explain the failure of the enzyme to bind or catalyze the decarboxylation of 2-thioOMP, with its bulkier sulfur atom which might not fit easily into the productive mode in which OMP would be bound. One should not dismiss the additional possibility, in our opinion, that the bulkiness of sulfur may force 2-thioOMP into a form in which the glycosidic bond is rotated in some another direction that does not correspond to either of the extremes occupied by UMP and its derivatives.

GROUND STATE DESTABILIZATION VERSUS TRANSITION STATE STABILIZATION

In principle, an enzyme can lower the energy of activation of a reaction by lowering the free energy of the enzyme-substrate complex in the transition state, or by raising the free energy of the enzyme-substrate complex in the ground state (Figure 2). Accordingly, any factor that destabilizes the ES complex in the ground state without affecting the stability of ES in the transition state is expected to enhance the value of \( k_{\text{cat}} \). Thus, ground state destabilization could help to explain the very large rate enhancement produced by OMP decarboxylase (\( k_{\text{cat}}/k_{\text{non}} \approx 10^{17} \)), one of its more remarkable features. The proposal by Wu et al. that OMP decarboxylation involves destabilization of the enzyme-substrate complex in the ground state, by means of repulsive interactions between an aspartate residue in the active site and the substrate’s carboxylate group, was based on modeling OMP into a cavity whose principal features were suggested by the crystal structures of enzyme complexes with other ligands. Using similar methods of calculation, Warshel et al. have arrived at a different conclusion: that
the catalytic effect of OMP decarboxylase can be reproduced without invoking ground state destabilization, and that the substrate is actually stabilized in the ground state ES complex (82, 83). These authors propose that the enzyme’s active site is organized in such a way as to stabilize the transition state preferentially, through electrostatic interactions involving the charged residues of the active site. If equilibrium is assumed to be maintained, then from the difference between neutrality and pH 2 (the latter equivalent to the approximate $pK_a$ value of the carboxylic acid group of orotic acid derivatives), Warshel et al. estimate that the maximum advantage in free energy that could be gained from destabilization of the substrate in the ES complex is 7 kcal/mol. If the reactive carboxyl group were destabilized by more than 7 kcal/mol, a proton from bulk solvent could presumably find its way between the mutually repulsive carboxylate groups. Considering that the free energy of activation for the uncatalyzed reaction approaches 39 kcal/mol, this level of substrate destabilization could account for only a minor part of the overall rate enhancement.

The differences of interpretation between Warshel and Wu stem from a difference in the choice of reacting systems. Wu et al. compare the decarboxylation of an orotate moiety in water with the same reaction at the active site of the enzyme. In contrast, Warshel & coworkers compare the decarboxylation of an orotate-lysine ion pair, in solution and within the active site. Using that modified reactant pair, Warshel et al. observed destabilization between the two aspartate residues within the protein, rather than destabilization between the aspartate residue and the reactive carboxyl group of the substrate. According to the Warshel model, the price of destabilization can be considered to have been invested in the energetics of protein biosynthesis and folding, rather than in the binding of the substrate’s phosphoribosyl moiety. The presence of a lysine residue bridging the distance between the aspartate residues and the negatively charged oxygen atom of OMP is considered to stabilize the ES complex in the ground state, but to stabilize the ES complex in the transition state to a much greater extent.

**MECHANISTIC EVIDENCE FROM STRUCTURE AND MUTATION**

Before the crystal structure of ODCase was solved, Lys-93 of the yeast enzyme had been identified as essential for activity (60). In their ground state destabilization hypothesis, Wu & coworkers propose that enzyme interactions with the phosphoribosyl group of substrate OMP supply much of the binding free energy ($\sim 26$ kcal/mol) that stabilizes the ES complex, and that this may force the substrate’s carboxylate group into the vicinity of the carboxylate groups of the active site. To explore the consequences of that prediction, Miller et al. (55, 87) performed experiments involving substrate truncation and active site mutagenesis. Removal of Tyr-217 or Arg-235, which interact with the phosphoryl group
of BMP in the crystal structure, was found to reduce the values of $k_{cat}/K_m$ by factors of 3000-fold and 7300-fold, respectively. When the phosphoribosyl group of substrate OMP was simply deleted, $k_{cat}/K_m$ was reduced by more than 12 orders of magnitude, despite the fact that the substrate orotic acid must be able to fit into any active site that can accommodate OMP. It was possible to measure this “substituent effect,” the largest that appears to have been recorded for an enzyme reaction, using high concentrations of enzyme and orotate enriched with carrier-free $^{14}$C at the carboxylate group, because the rate of the uncatalyzed reaction is so slow ($t_{1/2} = 10^8$ years) at ordinary temperatures. These findings support the conclusion drawn from earlier experiments on human UMP synthase (85) that the phosphoribosyl group plays an essential role in determining the relative binding affinities of various ligands including the substrate.

New experimental evidence indicates that the role of the phosphoribosyl group in promoting catalysis can be understood to a large extent in terms of transition state stabilization. Effects of mutation on ligand binding affinities, measured with either the Y217A or the R235A mutant enzymes, are shown in Table 1. They indicate that attractive interactions between the enzyme and the substrate phosphoryl group become progressively more important as these ligands approach the transition state in structure, culminating in the transition state itself. Although not shown in this table, the same tendency is observed when residues contacting the ribosyl 2′- and 3′-OH groups are mutated to alanine (86). The results of both studies are consistent with the view that the ODCase active site 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. Paradoxically, the phosphoryl group, although it undergoes no chemical transformation, appears to be of decisive importance to the enzyme in distinguishing between the substrate in the ground state and the altered substrate in the transition state. Not only is ODCase a remarkably proficient protein catalyst, but also it manifests the effect of a remote binding determinant [Jencks’ “anchor” or “Circe” effect (17)] in a pronounced form. Moreover, binding discrimination appears to be reflected in a steadily increasing affinity (UMP < OMP < BMP < transition state) rather than in any overall destabilization of the enzyme-substrate complex in the ground state (Table 1).

### TABLE 1
Increase in dissociation constant for various ligands, resulting from 4 mutations to alanine at the active site of yeast ODCase. A large value implies that the corresponding side-chain makes a large contribution to ligand binding affinity (86, 87).

<table>
<thead>
<tr>
<th>Mutation:</th>
<th>Y217A/WT</th>
<th>R235A/WT</th>
<th>K59A/WT</th>
<th>D96A/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMP</td>
<td>17</td>
<td>9</td>
<td>1.7</td>
<td>nd</td>
</tr>
<tr>
<td>OMP</td>
<td>130</td>
<td>70</td>
<td>910</td>
<td>11</td>
</tr>
<tr>
<td>BMP</td>
<td>2000</td>
<td>2600</td>
<td>11000</td>
<td>nd</td>
</tr>
<tr>
<td>transition state</td>
<td>3000</td>
<td>7500</td>
<td>120000</td>
<td>&gt;2000000</td>
</tr>
</tbody>
</table>
To explore the function of the four charged residues located in the region of the active site where the reactive carboxyl group is expected to be bound, Miller et al. replaced each of these residues with alanine in the yeast enzyme (87). Mutation to alanine of either Lys-93 or Asp-91 reduced activity by more than $10^5$-fold, yielding proteins that were incapable of binding substrate at a level that could be detected by isothermal calorimetry. Substitution of alanine for either of the two remaining members of the charged quartet (Lys-59 or Asp-96) was found to reduce activity by a factor of more than $10^5$ in each case but both proteins retained part of the native enzyme’s affinity for substrate OMP (Table 1). The failure of the Asp-91 mutant to bind ligands with appreciable affinity is of special interest, because the side chain of that residue was postulated to be involved in ground state destabilization (72). According to that hypothesis, in its simplest form, removal of either of the mutually repulsive pair of carboxylate groups (Asp-91 and the 6-carboxylate group of the substrate) might have been expected to cause an increase in substrate binding affinity.

As we have seen, catalysis depends on the enzyme’s ability to bind the altered substrate in the transition state more tightly than it binds the substrate in the ground state. That difference in binding affinity need not imply, however, that the substrate in the ground state is thermodynamically destabilized with respect to the unbound substrate in aqueous solution. Figure 7 shows that the affinity of OMP decarboxylase for substrate OMP ($K_m = 7 \times 10^{-7}$ M) is actually higher than its affinity for product UMP ($K_i = 2.0 \times 10^{-4}$ M), which lacks the 6-carboxylate group. In making this comparison, it should be noted that UMP appears bound in the syn form, which is relatively uncommon in free solution, amounting to perhaps 1% (87). Thus, the affinities of OMP and UMP, in the forms in which they are bound, are probably similar. The similarity of these binding affinities, coupled with the finding that removal of the carboxylate group of Asp-91 does not enhance the enzyme’s affinity for OMP, do not seem to offer support for the view that the carboxylate group of bound OMP is under electrostatic stress because of its juxtaposition to Asp-91.

How does the active site architecture of ODCase allow such a high level of discrimination between the binding of the substrate in the ground state ($K_i = 10^{-7}$ M) and the binding of the altered substrate in the transition state ($K_{ts} < 10^{-23}$ M)? The crystal structure of yeast ODCase in complex with 6-hydroxyuridine 5’-phosphate (BMP) (Figure 9) suggests the presence of electrostatic attraction between the negatively charged oxygen atom, located at the C-6 position of this inhibitor, and the positively charged side chain of Lys-93. On the basis of that structural information, one might expect Lys-93 to be in a position to form favorable interactions with the negatively charged carboxylate group of substrate OMP in the ground state ES complex. In fact, the native enzyme binds OMP with substantial affinity. In contrast, the lysineless K93A mutant enzyme fails to bind OMP with measurable affinity as judged by isothermal titration calorimetry (87). The electrostatic environment of Lys-93 suggests one qualitative explanation of how ODCase might maximize the stabilizing effect of this
residue in the transition state while minimizing the interaction of Lys-93 with the substrate in the ground state. The placement of Lys-93 between two negatively charged aspartate residues probably elevates the pKₐ value of its protonated side chain to an abnormally high value, and the large discrepancy between that value and the pKₐ value (~2) of the carboxylic acid group of OMP suggests that interaction between Lys-93 and the substrate carboxylate group are likely to be relatively weak in the ES complex if they exist at all. With approach to the anionic transition state, interactions involving Lys-93 might be expected to become increasingly favorable, especially in a nonaqueous environment (89, 90).

The crystal structures of ODCases in complex with various ligands are remarkably similar. However, Figure 10 shows that the affinities of yeast ODCase for a series of competitive inhibitors span a range of 9 orders of magnitude. Some of the differences in affinity observed between these ligands seem to furnish strong indications that the active site may be able to adopt conformations that have yet to be discovered. The enzyme’s high affinities for the purine derivative XMP, and for a 6-thiocarboxamido derivative of UMP (90) (as contrasted with the enzyme’s weak affinity for the corresponding carboxamido analog) are especially startling in view of the active site’s exacting structural discrimination between pyrimidines. In particular, the enzyme’s failure to bind or act upon 2-thioUMP (51) or to bind the 6-aldehyde derivative of UMP, whose synthesis was published recently (94) (M. Groziak, B. G. Miller & R. Wolfenden, unpublished data). We consider it likely that the substantial binding affinity of OMP in the ground state conceals important repulsive or distortion effects that are relieved in the transition state but which have yet to be clearly identified. Thus, the structure of the enzyme-OMP complex is now of most pressing interest.

CONNECTIVITY EFFECTS AND THE EFFECTIVE CONCENTRATIONS OF BINDING DETERMINANTS AT THE ACTIVE SITE

We have seen that the phosphoribosyl group appears to serve as an anchor to orient the substrate in the active site for transition state stabilization and that it contributes a major part of the free energy of binding that is needed to produce the catalytic effect of ODCase. Its contribution can be analyzed in a different way.
by comparing the enzyme’s affinity for OMP in the transition state (OMP$^\ddagger$) with
the enzyme’s affinity for OMP in the transition state (OMP$^\ddagger$) cut in two pieces:
orotate in the transition state (O$^\ddagger$) and ribose 5-phosphate (55). The results of that
operation, shown in Figure 11, indicate that the effective concentration of the
phosphoribosyl group exceeds $10^8$ M in the transition state for decarboxylation
of OMP. Although this connectivity effect is numerically impressive, effects of
comparable magnitude have been observed in the binding of substrates and
transition state analogue inhibitors by adenosine deaminase (95) and cytidine
deaminase (96). Even more remarkable is the case of triosephosphate isomerase,
in which the binding energy of the nonparticipating phosphoryl group has
recently been shown to account for nearly all the rate enhancement that this
enzyme produces (97).

To explore the role of connectivity in catalysis by ODCase in a different way, it
is of interest to consider the results of experiments in which the enzyme rather than
the substrate is “cut” into two pieces. The results obtained with two pieces of
ODCase, consisting of the lysineless K93A mutant and a primary amine, lead to a
similar conclusion (18). Thus, the activity (and therefore the transition state affinity)
of the lysineless mutant is reduced by a factor of at least $10^5$. Moreover, concen-
trated aqueous methylamine fails to produce any detectable catalysis of the decar-
boxylation of 1-methylorotate, even at temperatures (170°C) at which spontaneous
decarboxylation is measurable. That observation places an upper limit on the second
order rate constant for catalysis of this reaction by methylamine and a lower limit on
the value of $K_{tx}$ for methylamine as a catalyst. The results of this cutting operation,
shown in Figure 12, indicate that the effective concentration of Lys-93 at the active
site of yeast ODCase is greater than $10^6$ M. That level of cooperativity, although
remarkable, is not unique. The results of making similar cuts in mandelate racemase
(98) and in cytidine deaminase (99) have yielded similar results.

In that connection, it is worth noting that the total amount of free energy available
for binding that can be estimated by simply adding up the contributions of each active
site interaction (as measured by single-site mutations) exceeds 45 kcal/mol (Figure
13) (87). That total greatly surpasses the actual binding energy in the transition state
(∼31 kcal/mol) that is needed to explain the catalytic proficiency of ODCase.
Viewed in the opposite sense, this difference confirms the very great importance of
connectivity effects at the active site, as a determinant of the very high affinity that
this enzyme achieves for the altered substrate in the transition state.

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**Figure 11** Cutting OMP: Comparison of the binding affinity of yeast ODCase for OMP
in the transition state for its decarboxylation, for orotate in the transition state for its
decarboxylation, and for ribose 5-phosphate as an inhibitor (55). The “effective concentra-
tion” of the substrate in the transition state, expressing the advantage of having its parts
properly connected, exceeds $10^8$ M. For examples of this effect in other enzymes, see
references (95) adenosine deaminase, (96) cytidine deaminase, and (97) triosephosphate
isomerase.
**OMP as a whole**

A → B

\[ K_{\text{AB}} \downarrow \]

\[ K_{\text{AB}} < K_A K_B \]

\[ K_{\text{tx}} = 4.4 \times 10^{-24} \text{ M} \]

**OMP in pieces**

A → B

\[ K_A \downarrow \quad K_B \downarrow \]

A = ribose 5-phosphate as a competitive inhibitor

B = orotate as a substrate

\[ k_{\text{cat}} / K_M \leq 2.5 \times 10^{-5} \text{ M}^{-1} \text{s}^{-1} \]

\[ k_{\text{non}} = 2.2 \times 10^{-16} \text{ s}^{-1} \]

\[ K_i = 8.0 \times 10^{-5} \text{ M} \]

\[ K_{\text{tx}} \geq 1.1 \times 10^{-11} \text{ M} \]

**effective concentration of phosphoribosyl group** > \( 2 \times 10^8 \) M

**connectivity effect** > 11 kcal/mol
Cutting ODCase: Comparison of the apparent binding affinities for OMP in the transition state for its decarboxylation by wild-type ODCase, for its decarboxylation by the lysineless mutant enzyme K93A (87), and for its decarboxylation by methylamine. The “effective concentration” of Lys-93 in the transition state, expressing the advantage of having the parts of ODCase properly connected, exceeds 10^7 M. For examples of this effect in other enzymes, see references (98) mandelate racemase and (99) cytidine deaminase.
Figure 13. Effects on transition state binding affinity of converting active site residues individually to alanine (55, 86, 87).
CONCLUSIONS AND PROSPECTS

How can the altered substrate in the transition state be bound by an enzyme with a dissociation constant, in water, of less than $10^{-20}$ M, and how can this affinity exceed that of the substrate in the ground state - structurally similar in many respects - by at least 15 orders of magnitude? Detailed answers to these questions are expected to vary from case to case. However, it seems reasonable to suppose that catalysis tends to occur in two stages.

First, access of substrates and egress of products occurs with such ease (Figure 1) as to imply that the native active site is usually open to easy access of substrate and release of products in their ground states. If a very rare or activated species of the substrate were needed for productive encounter, or the enzyme were closed to substrate access for a very large fraction of the time, then $k_{cat}/K_m$ would fall correspondingly short of the limit imposed by the rate of encounter.

Following substrate binding, forces of attraction between the substrate and the enzyme increase, allowing the active site to compete effectively with solvent water in binding a transition state that differs in structure from the substrate in the ground state. The effects of these forces might be maximized if an enzyme’s active site tended to surround the altered substrate in the transition state in such a way as to allow each of these interactions (unlike interactions with solvent) to reinforce the others. Numerous X-ray diffraction structures of enzyme complexes with transition state analogues have revealed that the active site tends to surround bound transition state analogue inhibitors in such a way as to remove them almost completely from solvent water.

For such a scenario to lower the free energy of activation, the benefit of increased contact must outweigh the cost of distorting the enzyme from its native, or open, structure. The cost of distorting the enzyme from its native open structure, which must be paid as part of this process, is expected to be minor if it involves a hinge-like movement of two parts that are otherwise well ordered. Of the forces of attraction used by enzymes, H-bonds and electrostatic attractions appear to figure prominently in enzyme-inhibitor structures. H-bonds are weak or negligible when considered individually in water, just as general acid-base catalysis (which can be regarded as the catalytic equivalent of H-bonding in the transition state) is relatively ineffective in reactions of simple molecules in water, but relatively effective at close range (92, 98). Moreover, the constraints imposed by formation of one “weak” bond greatly enhance the probability of forming a second, and such effects can raise the apparent concentration of the reaction partner to a very high level. Effective concentrations of $10^5$-$10^8$ M have been observed experimentally by making “cuts” in enzymes or substrates, then comparing the binding affinities of these “pieces” with those of the native enzyme or substrate in the transition state. Combined with the large binding contributions that have been observed for individual groups, e. g., $10^8$-fold for single -OH substituent (100), these levels of synergism appear to bring the transition state affinities of enzymes within reach of ordinary forces of attraction.
In addition to its high affinity for the altered substrate in the transition state, an enzyme must be able to “fend off” (or bind weakly) the substrate in the ground state, if it is to enhance the rate of reaction. That ability to avoid tight binding of the substrate in the ground state, sometimes termed “ground state destabilization,” seems especially remarkable in view of the fact that the ground state and transition state differ only slightly in structure. One simple means of achieving that objective, which may be particularly useful in lyase reactions that involve a reduction in the size of the substrate, is by steric hindrance. Steric effects of this kind seem likely to figure prominently in the action of orotidine 5’-phosphate decarboxylase, which produces one of the largest rate enhancements known. Moreover, the topological requirements of an effective catalyst are considerably more complex than the conventional binding scheme (Figure 2) suggests. For example, in any enzyme reaction, regardless of its mechanism, the path from the ground state ES complex to the transition state almost always differs in detail from the path from the ground state EP complex to the transition state (12).

The mechanism of action of this enzyme will not be understood before its binding affinities are explained in structural terms. We consider it likely that the relatively weak overall binding affinity of OMP in the ground state masks repulsive or distortion effects, yet to be recognized, which are relieved in the transition state. Thus, the structure of the enzyme-OMP complex is now of pressing interest, and major efforts are being made to obtain that information using mutant enzymes in which the enzyme-OMP complex is unreactive.

ACKNOWLEDGMENTS

We are grateful to Mark Snider and Stephen Bearne for supplying the information presented in Figure 1 (for which literature citations will be provided upon request). We thank Peter Guthrie, Weitao Yang, and many of the authors cited here, for helpful advice and unpublished information.

APPENDIX

Representative values of catalytic efficiency \(\left(\frac{k_{\text{cat}}}{K_m}\right)\) at 25 °C reported for enzymes of various types, as displayed in Figure 1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>(\frac{k_{\text{cat}}}{K_m}) (M(^{-1})s(^{-1}))</th>
<th>Rate-det.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>Superoxide</td>
<td>(7 \times 10^9)</td>
<td>Diffusion</td>
<td>1</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Fumarate</td>
<td>(1 \times 10^9)</td>
<td>Diffusion</td>
<td>2</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>Glyceraldehyde 3-phosphate</td>
<td>(4 \times 10^8)</td>
<td>Diffusion</td>
<td>3</td>
</tr>
<tr>
<td>(\beta)-lactamase</td>
<td>Penicillin</td>
<td>(1 \times 10^8)</td>
<td>Partly diff.</td>
<td>4, 5</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Substrate</td>
<td>$k_{\text{cat}}/K_m$ (M$^{-1}$s$^{-1}$)</td>
<td>Rate-det.</td>
<td>Ref.</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------------------------</td>
<td>----------------------------------------</td>
<td>--------------------</td>
<td>------</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>MocTrp o-nitrophenyl ester</td>
<td>$9 \times 10^7$</td>
<td>Partly diff.</td>
<td>6</td>
</tr>
<tr>
<td>OMP decarboxylase</td>
<td>Orotidine 5′-phosphate</td>
<td>$6 \times 10^7$</td>
<td>Not diff.$^a$</td>
<td>7, 8</td>
</tr>
<tr>
<td>Cytochrome c peroxidase</td>
<td>Hydrogen peroxide</td>
<td>$5 \times 10^7$</td>
<td>Not diff.</td>
<td>9</td>
</tr>
<tr>
<td>Phosphotriesterase</td>
<td>P-nitrophenyl phosphate</td>
<td>$5 \times 10^7$</td>
<td>Diffusion</td>
<td>10</td>
</tr>
<tr>
<td>Catalase</td>
<td>Hydrogen peroxide</td>
<td>$4 \times 10^7$</td>
<td>Partly diff.</td>
<td>11</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>4-nitrophenyl phosphate</td>
<td>$3 \times 10^7$</td>
<td>Diffusion</td>
<td>12</td>
</tr>
<tr>
<td>Lipoygenase-1</td>
<td>Linoleic acid</td>
<td>$3 \times 10^7$</td>
<td>Partly diff.</td>
<td>13</td>
</tr>
<tr>
<td>HIV protease</td>
<td>Peptide</td>
<td>$2 \times 10^7$</td>
<td>Not diff.</td>
<td>14</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>Adenosine</td>
<td>$1 \times 10^7$</td>
<td>Partly diff.</td>
<td>15, 16, 17</td>
</tr>
<tr>
<td>Staphylococcal nuclease</td>
<td>DNA, pH 9.5</td>
<td>$1 \times 10^7$</td>
<td>Diffusion</td>
<td>18</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>Acetyl thiocholine</td>
<td>$1 \times 10^7$</td>
<td>Diffusion</td>
<td>19</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Carbon dioxide</td>
<td>$7 \times 10^6$</td>
<td>Partly diff.</td>
<td>20, 21</td>
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<tr>
<td>Carboxypeptidase a</td>
<td>Furrylacryloyl-Phe-Phe</td>
<td>$7 \times 10^6$</td>
<td>Diffusion</td>
<td>22</td>
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<tr>
<td>Cytidine deaminase</td>
<td>Cytidine</td>
<td>$3 \times 10^6$</td>
<td>Not diff.</td>
<td>23</td>
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<tr>
<td>Ribonuclease T2</td>
<td>GpC</td>
<td>$2 \times 10^6$</td>
<td>Diffusion</td>
<td>24</td>
</tr>
<tr>
<td>Chorismate mutase</td>
<td>Chorismate</td>
<td>$2 \times 10^6$</td>
<td>Diffusion</td>
<td>25, 26</td>
</tr>
<tr>
<td>Mandelate racemase</td>
<td>Mandelate</td>
<td>$1 \times 10^6$</td>
<td>Partly diff.</td>
<td>27</td>
</tr>
<tr>
<td>ACP synthase</td>
<td>S-adenosylmethionine</td>
<td>$1 \times 10^6$</td>
<td>Diffusion</td>
<td>28</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>L-aspartate</td>
<td>$1 \times 10^5$</td>
<td>Not diff.</td>
<td>29, 30</td>
</tr>
</tbody>
</table>

$^a$ In this table, “Diffusion” indicates those enzymes for which $(k_{\text{cat}}/K_m)$ is considered to be limited by productive substrate binding, and “Not diff.” indicates reactions for which $k_{\text{cat}}/K_m$ appears to be limited by an internal step rather than by productive substrate binding.

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**LITERATURE CITED**

76. Deleted in proof
78. Deleted in proof
84. Deleted in proof
88. Deleted in proof
91. Deleted in proof
NOTE ADDED IN PROOF

In a recently reported OMP complex of a double mutant of the thermophilic enzyme, with both Asp-91 and Lys93 mutated to alanine, the scissile C-C bond was found to be bent out of the plane of the pyrimidine ring, toward a cavity into which the investigators had previously suggested that CO$_2$ might be ejected (101).