Circe Effect versus Enzyme Preorganization: What Can Be Learned from the Structure of the Most Proficient Enzyme?

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The idea that enzymes work by increasing the ground state (GS) free energy of the reacting fragments has been frequently advanced. The most popular form of this proposal has been related to Jencks' "Circe effect" where the binding of the nonreactive part of the substrate is supposed to "push" the reactive part into a destabilizing environment.[1] However, quantitative computer simulation studies and energy considerations[2, 3] led repeatedly to the conclusion that GS destabilization (GSD) cannot be a major contributor to the rate enhancement of enzymes.[4] This conclusion has been challenged by two recent studies,[5, 6] which found evidence for GSD by using different computational approaches. These works had immediate impact[7, 8] in part because they considered the molecular mechanism of the most proficient enzyme known to date: orotidine 5′-monophosphate decarboxylase (ODCase).[9] The paper by Lee and Houk,[5] which appeared before the crystal structure of ODCase was available, proposed that ODCase achieves its remarkable catalytic activity by placing the negatively charged orotate group in a nonpolar environment. This "desolvation" mechanism, which has been also implicated in other cases (see, for example, ref. [1]), was criticized by two of us[3] who pointed out that it reflected an incorrect thermodynamic cycle. In fact, in ref. [3] we argued that any enzyme with a significant rate enhancement works by placing its substrate in a very polar (rather than nonpolar) environment and stabilizing the corresponding transition state (TS).

Very recently, the structure of ODCase has been solved in breakthrough studies of four research groups.[6, 10–12] Other important studies on this issue have also appeared recently.[13, 14] These studies confirmed the presence of a very polar (salt-like) environment, but were interpreted by several groups[6, 11, 13] as evidence for a GSD. That is, it was concluded that the interaction between the orotate and the negatively charged groups in the ODCase active site (Figure 1) destabilizes the reactant state. In addition, this structural arrangement was taken[6] as a confirmation of the Circe effect, in which the assumed very strong binding of the phosphoribosyl group pulls the orotate to its unfavorable environment and the electrostatic repulsion is released in the transition state. This conclusion seems to be supported by the calculations of Wu et al.[6] who suggested that the enzyme works by applying "electrostatic stress" on the GS of the substrate. This proposal has gained immediate approval in some circles (as discussed in ref. [8]), where it was accepted as verification of the elusive GSD mechanism. However, despite this excitement it seems to us (see below) that the analysis of Wu et al. cannot be considered as a demonstration of a GSD effect.

In order to analyze the actual information from the ODCase system we have to ask what was really found. First, most proponents of the GSD mechanism have emphasized that two aspartate residues (presumably negatively charged) are positioned near the carboxylate group of the substrate (Figure 1). The resulting electrostatic repulsion is assumed to lead to a large GSD. What is missing in this analysis is the stabilizing effect of Lys72, which is closer to the orotate than the

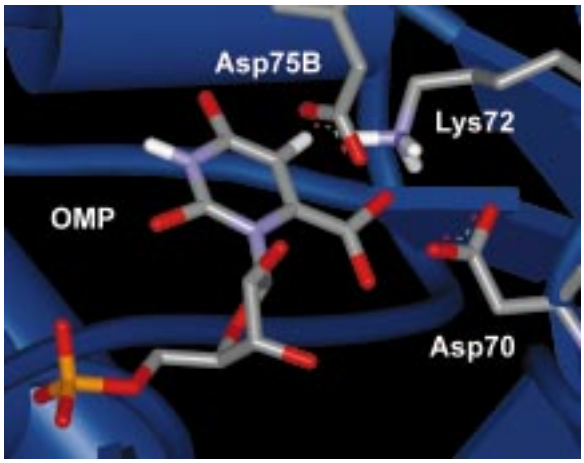

Figure 1. *The active-site region of ODCase. The presented structure is based on the crystal structure of ODCase with a TS analogue (PDB entry 1DV7), in which the TS analogue was converted into orotidine 5′-monophosphate (OMP) and the ODCase – OMP complex was relaxed by a molecular dynamics calculation.*

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aspartates (Figure 1). Thus, it is essential to analyze the structural information by careful free energy calculations before deciding whether or not there is a GSD effect. The calculations of Wu et al.[6] provided a significant step in this direction. They impressively reproduced the reduction of the activation barrier for the decarboxylation ($\Delta\Delta g^{\neq}$) by using the calculated activation barriers in the protein ($\Delta g^{\neq}_{cat}$) and in water ($\Delta g^{\neq}_{W}$) [Eq. (1)].

$$\Delta\Delta g^{\neq} = \Delta g^{\neq}_{cat} - \Delta g^{\neq}_{W} \quad (1)$$

Such calculations alone, however, say nothing about the presence or absence of GSD effects. To attribute the calculated $\Delta\Delta g^{\neq}$ value to GSD or TS stabilization it is essential to compare the free energy of binding in the ground state ($\Delta G_{bind,GS}$) and in the transition state ($\Delta G_{bind,TS}$) (see Figure 1 of ref. [3]). Then, in order for the observed rate enhancement to reflect a GSD effect, $\Delta G_{bind,GS}$ and $\Delta G_{bind,TS}$ should satisfy the relationships expressed in Equations (2) and (3).

$$\Delta G_{bind,GS} \approx -\Delta\Delta g^{\neq} \approx 23\ \text{kcal mol}^{-1} \quad (2)$$

$$\Delta G_{bind,TS} \approx 0 \quad (3)$$

However, Wu et al.[3] obtained almost equal positive values for $\Delta G_{bind,GS}$ and $\Delta G_{bind,TS}$ considering, respectively, the binding of the orotate part of the substrate (denoted here as S^-) and the corresponding transition state ($S^{-\neq}$). Consequently, they could not reproduce significant values of $\Delta\Delta g^{\neq}$ from their binding energies. This apparent paradox, which was overlooked by many readers, seems to reflect the fact that it is much harder to obtain converging results by evaluating ΔG_{bind} than $\Delta g^{\neq}$. In particular, since we deal with highly charged systems it is essential to have a proper treatment of long-range electrostatic effects.

A more systematic treatment of the long-range electrostatic effects was an important part of a recent work,[15] which revealed the following findings. First, the evaluation of $\Delta\Delta g^{\neq}$ by using the calculated values for $\Delta g^{\neq}_{cat}$ and $\Delta g^{\neq}_{W}$ produced the observed trend (i.e., $\Delta g^{\neq}_{cat} \approx 20\ \text{kcal mol}^{-1}$) in agreement with Wu et al.[6] Second and more importantly, the new calculations were able to reproduce the observed trend in $\Delta\Delta g^{\neq}$ from the magnitudes of $\Delta G_{bind,GS}$ and $\Delta G_{bind,TS}$ for S^- and $S^{-\neq}$, respectively. Here it was found[15] that $\Delta G_{bind,GS}$ is between -3 and $+4\ \text{kcal mol}^{-1}$, basically contradicting the GSD hypothesis. On the other hand, the catalysis was calculated to result from the TS stabilization (i.e., $\Delta G_{bind,TS}$ was around $-20\ \text{kcal mol}^{-1}$). Furthermore, it was found that a more consistent analysis leads to TS stabilization even when S^- is destabilized. That is, the actual reaction involves the process described in Equation (4).

$$\text{orotate}^- + \text{LysH}^+ \rightarrow \text{uracil} + \text{Lys} + CO_2 \quad (4)$$

This reaction includes a proton transfer from the protonated residue Lys 72 (LH^+) to S^-. This means that the reactive part considered in $\Delta G_{bind,GS}$ and $\Delta G_{bind,TS}$ should include the ($S^-LH^+ \rightarrow S'HL + CO_2$) system rather than only the ($S^- \rightarrow S'H + CO_2$) system. Now we have an entirely different picture than that obtained by including only S^- in the reacting region. Once we consider the true reactant state, which includes both the proton donor and the proton acceptor, we will obtain ground state stabilization rather than GSD by almost any computational model. Our calculations for the complete reacting system gave $\Delta G_{bind,GS} \approx -30\ \text{kcal mol}^{-1}$ and $\Delta G_{bind,TS} \approx -47\ \text{kcal mol}^{-1}$. Now (see Figure 2) the aspartate residues are preorganized in an optimal position to stabilize the dipole moment of the $[S^-LH^+]_p^{\neq}$ transition state. Since some readers might consider the selection of $[S^-LH^+]$ as the reactive part as being a semantic issue, it is important to emphasize that in proton transfer reactions both the proton donor and the acceptor represent integral parts of the reacting system. For example, this is the case in serine proteases, where all previous studies considered the proton acceptor (His 64) as a part of the reacting system.[2]

It might be also useful to comment on a proposal[12] that ODCase works by using a short, strong hydrogen bond (SSHB) between the orotate and Asp 70 (according to the notation used by Wu et al.[6]). First, the special role of the SSHB and related models is very problematic.[16] Second, and more specifically, our preliminary ab initio calculations of the mechanism described in ref. [12] produced a very large activation barrier for the reference reaction in water. In fact, the proposed hydrogen bond will stabilize the GS more than the TS.

Besides computer simulations, are there any other approaches we could use to assert the role of GSD in the rate

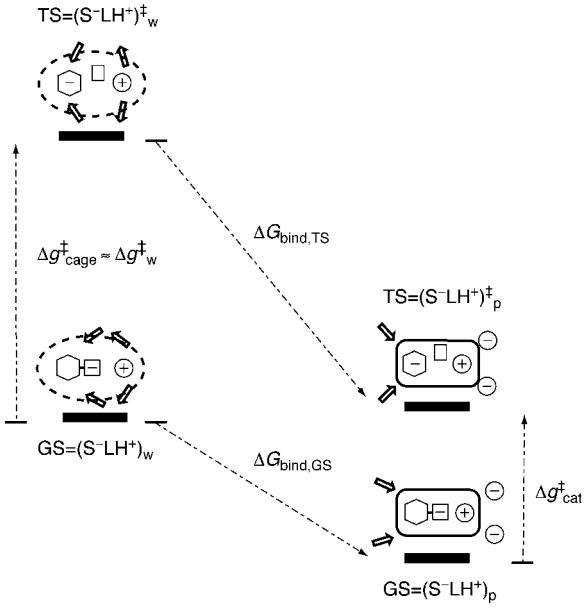

Figure 2. *The energetics of binding the reacting fragments in the GS and the TS of ODCase. S^- and LH^+ designate the orotate and Lys 72, respectively. The pyrimidine ring of the substrate and the carboxylate group (or CO_2) of the orotidine are described schematically by hexagons and squares, respectively.*

enhancement of ODCase? Here, general energy considerations based on the observed pK_a values and dissociation constants might provide additional insights. Let us assume for a moment that the catalytic effect is indeed due to GSD of S^- as a consequence of the electrostatic repulsion between negative charges. Such a major destabilization of S^- will lead (at equilibrium) to a new reactant state where the negatively charged substrate or the negatively charged protein residues will become protonated. Since the pK_a value of orotic acid is about 2, orotate cannot be destabilized by more than ca. 7 kcal mol^{-1} without being protonated by a bulk proton in an equilibrated enzyme – substrate (ES) complex. Alternatively, electrostatically "stressed" orotate or aspartate (p$K_a = 3.8$) can be protonated by a proton transfer from a protein residue. However, for true GSD we need to destabilize S^- by about 23 kcal mol^{-1}. Another problem with GSD and the corresponding Circe effect is that it requires an enormous free energy of binding of the phosphoribosyl part. That is, if the value of 23 kcal mol^{-1} for $\Delta\Delta g^{\neq}$ is due to destabilization of the reacting part then the observed total free energy of substrate binding of about -9 kcal mol^{-1} would require a contribution of -32 kcal mol^{-1} from the phosphoribosyl part of the substrate. Such a free energy of binding is without precedent. Furthermore, we now have direct estimates of the phosphoribosyl binding energy that is about -15 kcal mol^{-1}[10, 17] rather than -32 kcal mol^{-1}.

It is important to note that mutations of the crucial Asp residues should help in determining whether or not we have any GSD. According to Figure 2 of ref. [16] and Figure 12 of ref. [15], if such mutations will increase both $|\Delta G_{bind}|$ and $\Delta g^{\neq}$ we have a GSD mechanism; on the other hand, if $|\Delta G_{bind}|$ will decrease or stay constant and $\Delta g^{\neq}$ will increase we have a TS stabilization mechanism.

In summary, the exciting solution of the structure of ODCase has given us the chance to explore the origin of what is perhaps the highest proficiency of any enzyme known. Despite the great temptation to ascribe the action of this enzyme to the Circe effect a more careful analysis does not support this proposal. Yes, we have here "electrostatic stress" but it is the stress between the preorganized enzyme groups (the aspartate residues), rather than between the enzyme and the substrate. This "stress" is the previously proposed preorganization energy put forward by one of us[18] as the origin of enzyme catalysis.

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