Communication

Just a Near Attack Conformer for Catalysis (Chorismate to Prephenate Rearrangements in Water, Antibody, Enzymes, and Their Mutants)

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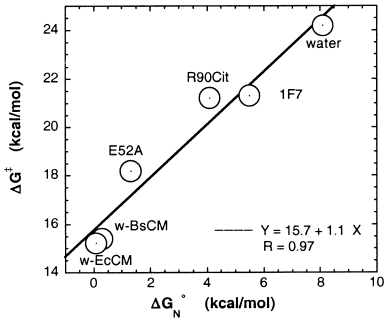

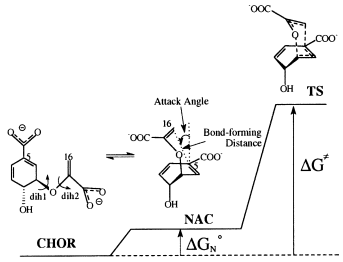

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Just a Near Attack Conformer for Catalysis (Chorismate to Prephenate Rearrangements in Water, Antibody, Enzymes, and Their Mutants)

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The definition of the rate enhancement for a single substrate enzyme is the ratio of the first-order rate constants k_{cat}/k_o, where k_{cat} pertains to the conversion of enzyme·substrate complex (E·S) → enzyme·transition state complex (E·TS) and k_o pertains to the conversion of S → TS in water at pH 7. To understand just how good an enzyme is as a catalyst, and why, the mechanisms of both the enzymatic and the water reactions must be determined. We have examined a series of single-substrate intramolecular reactions, which do not involve covalent enzyme−substrate intermediates.[1−3] Such intramolecular rearrangements in both water and enzyme are first order, and the binding of the TS in E·TS is devoid of covalent character. Thus, the kinetic importance of both substrate and enzyme conformations in the E·S and E·TS complexes can be examined, and these can be compared to S and TS in the spontaneous reaction in water.

In covalent bond formation, regardless of the environment, nucleophilic and electrophilic atoms must come together at a van der Waals distance and at an angle approximating that in the TS. We term such ground-state conformers as near attack conformers, or NACs (eq 1).

$$\underset{\text{ground state}}{S \overset{\Delta G_N^\circ}{\rightleftharpoons} NAC} \xrightarrow{\Delta G_{TS}^{\ddagger}} [TS] \qquad (1)$$

$$\Delta G^{\ddagger} = \Delta G_N^\circ + \Delta G_{TS}^{\ddagger} \qquad (2)$$

The free energy for formation of a NAC (ΔG_N°) can be determined quantitatively either by calculating the mole fraction of NAC relative to the total ground-state conformers during a molecular dynamics (MD) simulation or by use of free energy calculation methods. Once ΔG_N° is obtained, the activation energy on going from NAC to the TS ($\Delta G_{TS}^{\ddagger}$) can be calculated by subtracting ΔG_N° from the total activation energy ($\Delta G^{\ddagger}$) (eq 2), where the best determination of $\Delta G^{\ddagger}$ is from the experimental rate constant. The advantage of the enzymatic reaction over the water reaction ($\Delta\Delta G^{\ddagger} = RT \ln(k_{cat}/k_o)$) can be partitioned into $\Delta\Delta G_N^\circ$ and $\Delta\Delta G_{TS}^{\ddagger}$. The value of $\Delta\Delta G_N^\circ$ represents the kinetic advantage of enzyme as compared to water in the formation of NAC, and $\Delta\Delta G_{TS}^{\ddagger}$ represents the advantage in conversion of NAC to the TS.

The mechanisms of conversion of chorismate to prephenate (Scheme 1) by enzymes and catalytic antibodies have drawn much attention. This intramolecular rearrangement involves no covalent intermediates, and the rate constants for both enzymatic reactions (k_{cat}) and the nonenzymatic reaction in water (k_o) are known.[4−6] NACs for the chorismate → prephenate reaction are defined as conformers which have the two reacting moieties (C5 and C16) within van der Waals contact distance (≤3.7 Å) at an approaching angle (see Scheme 1) of ≤30° and the electron π-orbital of C16 pointing at C5.[7−9] In our previous MD studies on the conversion of chorismate to prephenate by *E. coli* chorismate mutase (EcCM), we estimated that $\Delta\Delta G_N^\circ = 7-8$ kcal/mol and $\Delta\Delta G_{TS}^{\ddagger} = 1-2$ kcal/mol out of $\Delta\Delta G^{\ddagger} = 9$ kcal/mol.[10] From this, the kinetic advantage of the enzymatic reaction, as compared to the reaction in water, principally originates from the greater mole percentage of ground-state conformers being NACs in the enzymatic reaction. This is supported by the Karplus group in their computational studies on the mutants of *B. subtilis* chorismate mutase (BsCM).[11] These findings, however, are in stark contrast to the generally accepted tenet that the efficiency of enzymatic reactions is due to enzyme preferentially stabilizing the TS as compared to the ground state.

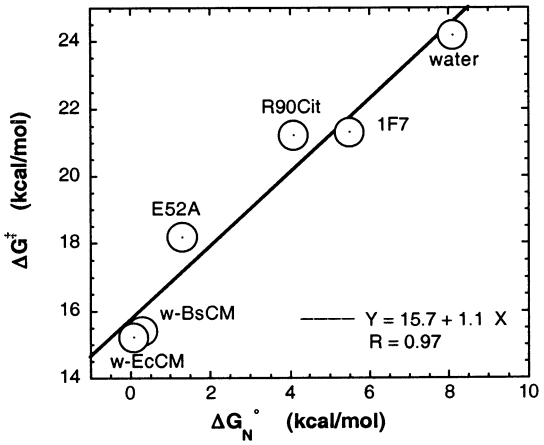
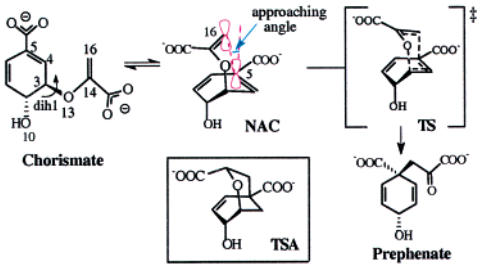


Figure 1. Plot of the computed free energy of NAC formation (ΔG_N°) and the experimentally determined activation energy ($\Delta G^{\ddagger}$) for the Claisen rearrangement of chorismate. Circles are of 1 kcal/mol diameter, which represent the computational error range. The six values of ΔG_N° in water, 1F7, R90Cit, E52A, w-BsCM, and w-EcCM were calculated by use of thermodynamics integration methods implemented in CHARMM v.27.[15] The dihedral angle dih1 (C4−C3−O13−C14, see Scheme 1) was chosen as an internal coordinate along which free energy derivatives were calculated. The goodness of fit (slope 1.1, correlation coefficient 0.97) is validation of these methods.

Scheme 1. Claisen Rearrangement of Chorismate



Information from the X-ray crystal structures of enzymes complexed with "transition state" analogue (TSA, Scheme 1),[12,13] as well as from kinetic studies of the mutant enzymes,[5,6] supports the necessity of a positively charged moiety (Arg90 in BsCM and Lys39 in EcCM) next to the chorismate ether O13, as well as a negatively charged glutamate (Glu78 in BsCM and Glu52 in EcCM)

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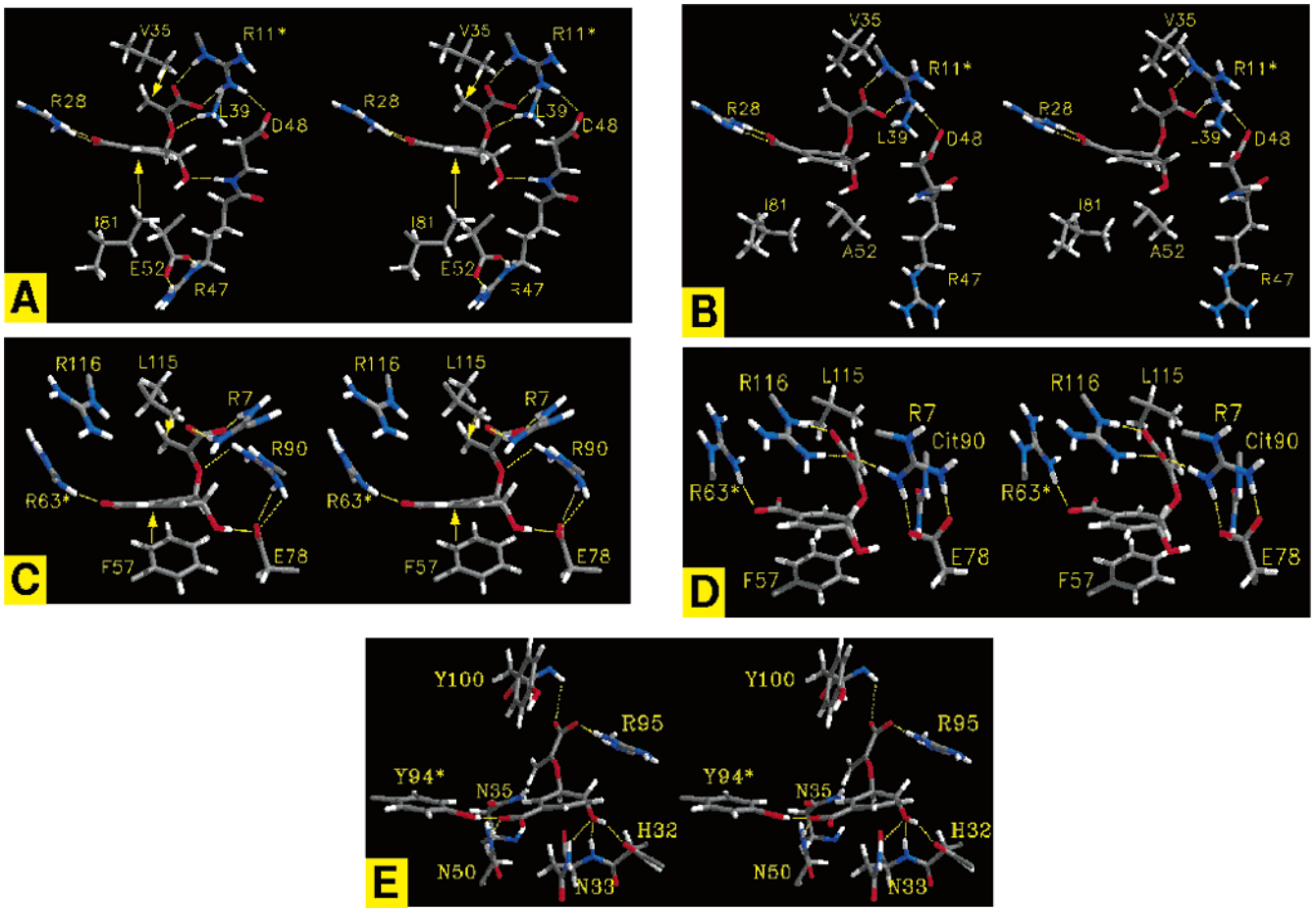

Figure 2. Stereoviews of chorismate at the active sites of (**A**) w-EcCM, (**B**) E52A mutant of EcCM, (**C**) w-BsCM, (**D**) R90Cit mutant of BsCM, and (**E**) the catalytic antibody 1F7. The structures **A**−**E** are the most stable geometries as they were taken from the free energy minimum points. Yellow dotted lines represent electrostatic interactions, whereas yellow arrows represent van der Waals contacts. In the two wild-type enzymes (**A** and **C**), the two carboxylates of chorismate are held by R28 and R11* in w-EcCM, and R63* and R7 in w-BsCM. (Notations * designate residues from other subunits of a protein.) In addition, in both wild-type enzymes, the side-chain vinyl group and the ring of chorismate are confined in a small space by having van der Waals contacts with hydrophobic protein residues. In w-EcCM, the V35···C16 and I81···C5 distances are 3.7 ± 0.2 and 3.8 ± 0.4 Å, respectively. In w-BsCM, the contact distances of L115···C16 and F57···C4 are 3.8 ± 0.3 and 3.7 ± 0.3 Å, respectively. In the E52A mutant (**B**) of EcCM, the bottom part of the active site is solvent-accessible via a newly formed water channel. This channel is closed in w-EcCM (**A**) due to the E52···R47 interaction. The loss of this interaction also makes backbone structures near R47 very mobile, resulting in weakening of several electrostatic interactions between protein and chorismate (i.e., D48(NH)···O10 and L39(NZ)···O13). In the R90Cit mutant (**D**) of BsCM, the mutation results in significant reorganizations of the active site residues. In w-BsCM (**B**), the R90 guanidine group is placed near O13 by interacting with E78. Elimination of charge for the R90Cit mutant makes a charge imbalance near E78, which attracts the nearby positively charged R7. This results in the chorismate carboxylate interacting with R116 instead of R7, forming a nonreactive chorismate conformer. The catalytic antibody (**E**) has R95 tightly holding the chorismate side-chain carboxylate, and N33 and H32 holding the ring hydroxyl group. The ring carboxylate of chorismate interacts with Y94*, but the interaction is very weak and exists only when the chorismate is in an extended nonreactive conformation. Being extensively exposed to bulk solvent, chorismate, as well as protein residues, easily changes conformations in 1F7.

near the chorismate O10 hydroxyl group. These observations have been interpreted as establishing that stabilization of the polar TS is the most important feature of the mechanism of chorismate mutases.

To examine further the contributions of ground-state NAC formation and TS stabilization to the chorismate → prephenate catalysis, we have performed free energy calculations of ΔG_N° for the reaction in water and by wild-type BsCM (w-BsCM), wild-type EcCM (w-EcCM), the mutant obtained by citrulline substitution for Arg90 of BsCM (R90Cit), the mutant obtained by alanine substitution for Glu52 of EcCM (E52A), as well as by the catalytic antibody 1F7[14] (Figure 1).

Figure 1 shows a plot of the computationally determined ΔG_N° versus the experimentally determined[4−6] $\Delta G^\ddagger$ for the Claisen rearrangement of chorismate in the six systems. The linear fit for these six data sets has a slope of 1.1 and a correlation coefficient of 0.97. *This shows that the ability of the systems to create NACs in the ground state is the major determinant of the free energy of activation of the Claisen rearrangement of chorismate.*Values of the free energies for the conversion of NAC to the TS ($\Delta G_{TS}^\ddagger$) in the six systems studied are presented in Table 1. As is indicated by the slope of ∼1, $\Delta G_{TS}^\ddagger$ is constant at ∼16.1 ± 1.0 kcal/mol, regardless of the system. At most, the free energy advantages for w-BsCM and w-EcCM over water are only 1.0 kcal/mol on going from NAC to the TS. This modest 1 kcal/mol advantage may represent a preferential stabilization of the TS over NAC by the charged residues in enzymes that is in excess of the stabilization afforded by polar water molecules.

Table 1. Values (kcal/mol) of Free Energies Used in Figure 1

	$\Delta G^\ddagger$	−	ΔG_N°	=	$\Delta G_{TS}^\ddagger$
water	24.2		8.1		16.1
1F7	21.3		5.5		15.8
R90Cit	21.2		4.1		17.1
E52A	18.2		1.3		16.9
w-BsCM	15.4		0.3		15.1
w-EcCM	15.2		0.1		15.1

Why are NACs unfavorable in water, and what features of enzymes enhance formation of NACs at the active site? By its geometrical definition, NAC formation for chorismate → prephenate requires proximity of the two carboxylates within the chorismate molecule. In water, this is very unfavorable due to the electrostatic repulsion between the two carboxylates.[16,17] This problem is resolved in the wild-type enzymes of EcCM and BsCM by having the two carboxylates form two tight salt bridges with two well-placed positively charged arginines (Figure 2A and 2C). Once the electrostatic interactions of arginines and carboxylates are formed, the conformation of enzyme-bound chorismate approximates NAC structures. Furthermore, in both w-EcCM and w-BsCM, hydrophobic bulky amino acid side-chain groups above the chorismate side-chain vinyl group and below the ring confine the free space between C5 and C16, resulting in the high populations of NAC at the active sites. In the E52A mutant (Figure 2B) of EcCM, many of the geometrical features in w-EcCM remain unaltered, and the stability of NAC at the active site is decreased only by 1.2 kcal/mol. Such small differences are hard to rationalize. However, a few changes are observed for the mutant, such as entry of solvent to the active site through a newly formed water channel and weakening of two hydrogen bonds to the hydroxyl O10 and the ether O13 of chorismate (Figure 2B).

Unlike the two wild-type enzymes and E52A, the mutant R90Cit and the antibody 1F7 do not exhibit much in support of NAC structures. In R90Cit (Figure 2D), the side-chain carboxylate of chorismate is placed in a different position by interacting with Arg116, instead of with Arg7 as in w-BsCM. In this altered interaction mode between the enzyme and chorismate, the distance between C5 and C16 of chorismate is 4.5 ± 0.2 Å, as compared to 3.5 ± 0.3 Å in w-BsCM. In 1F7 (Figure 2E), the side-chain carboxylate forms a salt bridge with an arginine, but the ring carboxylate is exposed mainly to solvent and exhibits much conformational flexibility. In the most stable form in 1F7 (Figure 2E), the chorismate side-chain vinyl group is placed away from the ring and is buried in the protein. The previously identified TRNOES between the C16 hydrogen and the ring hydrogens near C5,[18] appear to be due to indirect transfer via surrounding protein residues.

The linear free energy plot of ΔG_N° versus $\Delta G^\ddagger$ (Figure 1) with a slope of ~1.0 for the intramolecular reaction of chorismate → prephenate (Scheme 1) recapitulates the very same slope of ~1.0 in the plot of ΔG_N° versus $\Delta G^\ddagger$ for the intramolecular formation of cyclic anhydride from dicarboxylic acid monophenyl ester in the absence of any protein catalyst in aqueous solution.[19,20] Structures of monoesters determine the ease of NAC formation. Our current studies on the Claisen rearrangement, along with our previous studies on intramolecular anhydride formation, clearly demonstrate that, for some reactions, efficiency of forming reactive conformers in the ground state can be the most important kinetic contribution. This same principle can be applied to intramolecular reactions in both organic chemistry and enzymology.

The predominant importance of the ground-state conformational effect that we find in the chorismate → prephenate reaction is, however, not an attribute of enzyme reactions in general. Thus, in our comparison of the two intramolecular rearrangement reactions catalyzed by EcCM and chalcone isomerase,[21] it was found that, although ground-state contribution is of overwhelming importance in EcCM catalysis, this is not so in the catalysis by chalcone isomerase. Chalcone isomerase owes its rate enhancement primarily to features characterized as TS binding, with some minor contribution of ground-state NAC formation. It turned out that these enzymes have done what one would have expected to exhibit useful rates of reactions in excess of the water reactions. For the chorismate rearrangement, NAC is very rarely formed in water, such that enzyme could promote the reaction rate simply by providing an active site environment where NAC is favorable, whereas in chalcone isomerase, NAC is readily formed even without enzymatic assistance so that it is necessary that the enzyme reduces the energy gap between NAC and the TS to reach the biologically relevant reaction rate. Thus, one could not do better than to bet on the proposal that (i) enzymes utilize both NAC and TS stabilization in the mix required for the most efficient catalysis; and (ii) the importance of ground-state reactive conformer formation in determining the rate enhancement by an enzyme is inversely dependent upon the ease of forming the reactive conformer without enzymatic assistance in aqueous solution.

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