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A Comparative Study of Claisen and Cope Rearrangements Catalyzed by Chorismate Mutase. An Insight into Enzymatic Efficiency: Transition State Stabilization or Substrate Preorganization?

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Abstract: In this work we present a detailed analysis of the activation free energies and averaged interactions for the Claisen and Cope rearrangements of chorismate and carbachorismate catalyzed by *Bacillus subtilis* chorismate mutase (BsCM) using quantum mechanics/molecular mechanics (QM/MM) simulation methods. In gas phase, both reactions are described as concerted processes, with the activation free energy for carbachorismate being about 10−15 kcal mol^{-1} larger than for chorismate, at the AM1 and B3LYP/6-31G* levels. Aqueous solution and BsCM active site environments reduce the free energy barriers for both reactions, due to the fact that in these media the two carboxylate groups can be approached more easily than in the gas phase. The enzyme specifically reduces the activation free energy of the Claisen rearrangement about 3 kcal mol^{-1} more than that for the Cope reaction. This result is due to a larger transition state stabilization associated to the formation of a hydrogen bond between Arg90 and the ether oxygen. When this oxygen atom is changed by a methylene group, the interaction is lost and Arg90 moves inside the active site establishing stronger interactions with one of the carboxylate groups. This fact yields a more intense rearrangement of the substrate structure. Comparing two reactions in the same enzyme, we have been able to obtain conclusions about the relative magnitude of the substrate preorganization and transition state stabilization effects. Transition state stabilization seems to be the dominant effect in this case.

1. Introduction

Chorismate mutases catalyze the pericyclic Claisen rearrangement of chorismate to prephenate, an important step in the biosynthesis of aromatic amino acids.[1] In aqueous solution, this reaction proceeds in a single concerted step preceded by a conformational equilibrium among nonreactive and reactive chorismate structures (see Scheme 1). The reactive conformer presents the ring substituents into a pseudodiaxial disposition, while the one having a pseudodiequatorial arrengement is the most stable in aqueous solution.[2−6] From the former conformer, the chairlike transition state (TS) leading to prephenate can be reached in a single step. In the enzyme, the active site keeps the flexible substrate into its reactive conformation.[7−10] The reaction step seems to be favored by means of electrostatic interactions.[9] In this sense, a positively charged arginine residue (Arg90) is thought to play a decisive role in stabilizing the reaction transition structure.[11−13]

Chorismate mutases are thus an excellent example to analyze and quantify two different aspects which are expected to contribute to enzymatic efficiency: (i) the constraint effect on the reactants, restraining flexible molecules into a conformation

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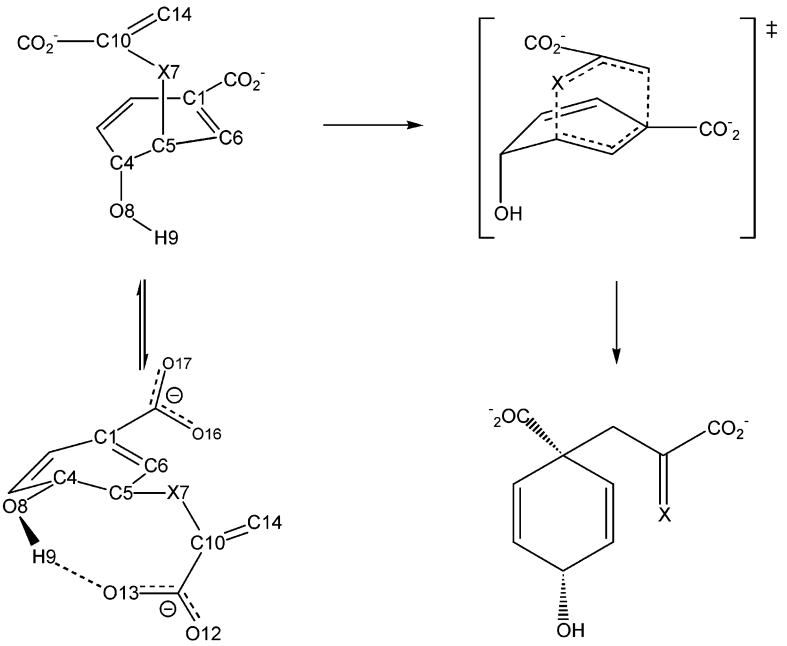
Scheme 1



X = O Claisen rearrangement of chorismate to prephenate.

X = CH_2 Cope rearrangement of carbachorismate to carbaprephenate.

closer to the transition structure,[10,14−16] here named as the substrate preorganization effect and (ii) the preferential electrostatic stabilization of the transition structure with respect to the same process in solution,[17] which is accompanied by a minor change in the environment as the reaction proceeds and, for this reason, is also known as the reorganization effect. Many theoretical studies have been recently devoted to the debate about the prevalence of substrate preorganization or transition state stabilization in enzyme catalysis.[15,17−20] We recently presented a theoretical study of these two aspects in *Bacillus subtilis* chorismate mutase (BsCM), showing that they are related factors having a common origin in the enzyme structure.[9] The active site is designed to stabilize electrostatically the transition state relative to the ground state, and as a consequence, it also favors those reactant structures which are more similar to the TS of the catalyzed reaction, the reactive reactants. To accommodate reactant structures differing from the transition structure, the enzyme should be deformed, resulting in an energy penalty.

A new experiment has been recently carried out by Hilvert et al.[21] to investigate these aspects of enzyme catalysis in chorismate mutases. They studied the Cope rearrangement of carbachorismate to carbaprephenate (see Scheme 1) and tested the catalytic activity of BsCM against this reaction. In the carba analogues, the ether oxygen is substituted by an apolar methylene group, and if they were expected to bind BsCM in a similar orientation to that of chorismate and prephenate, this new methylene group would be juxtaposed with Arg90. To isolate this destabilizing factor, Hilvert et al. also tested the activity of R90G and R90A variants of BsCM and the catalytic antibody 1F7, which lacks Arg90. No significant catalytic activity was found in any case, concluding that an active site which is designed to constrain the substrate in the reactive conformation (this is, presenting preorganization effect) but lacking specific electrostatic interactions (reorganization effect) is not enough to overcome the energy barrier of the Cope rearrangement.

In this work, we present a comparative analysis of both processes in BsCM: the Claisen rearrangement of chorismate to prephenate and the Cope rearrangement of carbachorismate to carbaprephenate. The activation free energy, specific interactions, and constraint of reactant structures are here presented for the Cope process and compared with the results of the Claisen reaction. The role of the enzyme is analyzed in terms of the key interactions established with the substrates in the Michaelis Complex and TS of both reactions. Therefore, we can rationalize the relative efficiency of the enzyme in terms of preorganization of the substrate and/or reorganization of the enzyme.

2. Methodology

Calculations in gas phase have been carried out at the AM1[22] and B3LYP/6-31G*[23] levels using the Gaussian98 package of programs.[24] Berny's[25] algorithm was used in both minima and transition structures localization; meanwhile, characterization was made by inspection of the Hessian matrix. Contributions

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to free energies have been obtained using standard formulas. In the case of semiempirical calculations, it must be taken into account that some terms can be then doubly counted. To compare similar quantities in different media, the activation and reaction free energies are calculated from the reactant and product states directly obtained from reaction path following a start at the transition state.

To study the Claisen and Cope rearrangements in solution and in the enzyme active site, we used the quantum mechanics/molecular mechanics (QM/MM) approach. In this methodology only, a small part of the system (typically those atoms directly involved in the bond breaking/forming process) is described at a QM level, while for the rest a MM energy function is used. This methodology was already reported in 1976, and since then, it has been successfully employed to study many chemical reactions in solution and in enzymes.[26−33]

QM/MM potential energy surface (PES) exploration of the enzymatic and aqueous solution rearrangements has been carried out using Charmm24b2[34] and Grace[35] programs. A putative transition structure for the carbachorismate rearrangement was obtained from a modified transition structure for the chorismate rearrangement at the BsCM active site.[36] In both cases, the reacting system has been described by means of the AM1 semiempirical MO method (24 atoms for the chorismate and 26 atoms for the carbachorismate). The transition structure is located and characterized defining a reduced Hessian matrix in which all the coordinates of the QM subsystem are explicitly included.

Exploration of the PES around the TS region provides an adequate starting point for molecular simulations, ensuring that the enzyme−substrate complex is in an active form, that is, ready for the reaction. The potentials of mean force for both rearrangements have been traced along the reaction path, using the Umbrella Sampling approach and the weighted histogram analysis method[37] as implemented in the Dynamo program.[38] The reacting system is also described by means of the AM1 semiempirical MO method, while the rest of the system, enzyme environment, and/or water molecules were described using the OPLS[39] and TIP3P[40] potential. The definition of the distinguished internal coordinate used to build the PMF was based on the data obtained in the PES exploration.[36] As could be expected, the best choice was the antisymmetric combinations of the distances describing the breaking and the forming bonds, i.e., $d_{C1,C14}-d_{C5,X7}$ (see Scheme 1). To obtain some representative averaged properties, additional QM/MM dynamical simulations in the enzyme and in aqueous solution were performed at the maximum and the reactant minimum of the PMFs. All the simulations were carried out using the NVT ensemble at a temperature of 300 K with periodic boundary conditions. A smoothed cutoff radius of 13.5 Å was employed.

Table 1. Distances of Transition Structures and Activation and Reaction Energies for the Claisen (X = O) and Cope (X = C) Rearrangements in Gas Phase at the AM1 and B3LYP/6-31G* Levels[a]

	chorismate		carbachorismate	
	B3LYP/6-31G*	AM1	B3LYP/6-31G*	AM1
$d_{C5,X7}$	2.275	1.854	2.306	2.115
$d_{C1,C14}$	2.719	2.164	2.254	2.121
$\Delta E^{\ddagger}$	44.1	52.7	57.8	63.5
$\Delta G^{\ddagger}$	41.1	52.1	55.9	62.8
ΔG_r	2.7	−7.9	14.4	20.9

[a] Bond distances in Å and energies in kcal mol^{-1}.

3. Results

Potentials of Mean Force. Chorismate and carbachorismate rearrangements take place in a single reaction step through a cyclic transition structure, as shown in Scheme 1. The relative position of the hydroxyl group with respect to the ring determines two possible reaction paths.[36] Here, we restrict to the outward orientation which is the preferred one in the BsCM active site.[36] Distances of the C5−X7 broken bond and the new C1−C14 bond in the gas-phase transition structure, together with their corresponding energy and free energy barriers, are given at the AM1 and B3LYP/6-31G* levels in Table 1. At both levels, chorismate rearrangement is described as a concerted but asynchronous process, in which C−X cleavage is more advanced than C−C bond formation in the transition structure. For carbachorismate, a more synchronous transition structure is obtained. The free energy barriers associated to the chorismate rearrangement are 10.7 and 14.8 kcal/mol lower than those for carbachorismate at the AM1 and B3LYP/6-31G* levels, respectively. Thus, the Cope reaction has a much larger intrinsic activation free energy. This is related to the fact that the carbachorismate rearrangement is much more endothermic than that for chorismate. In both cases, AM1 calculations show a clear trend to overestimate the energy barriers with respect to density functional calculations. We will correct this error in our AM1/MM simulations.

Activation free energies and averaged C1−C14 and C5−X7 bond distances of the transition structures for chorismate and

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Table 2. Averaged Distances of Transition Structures and Free Energy Barriers for the Claisen (X = O) and Cope (X = C) Rearrangements in BsCM Obtained by Means of the AM1/MM Calculations[a]

	chorismate		carbachorismate	
	aq solution	BsCM	aq solution	BsCM
$\langle d_{C5,X7} \rangle$	1.83	1.78	1.70	1.87
$\langle d_{C1,C14} \rangle$	2.14	2.10	1.74	1.90
$\Delta G^{\ddagger}$	37.9	29.3	48.6	43.0
$\Delta G^{\ddagger}_{corr}$	26.9	18.3	41.7	36.1

[a] Bond distances in Å and energies in kcal mol^{-1}. Corrected free energies are based on gas-phase free energy calculations.

Table 3. Differences in the Activation Free Energies (kcal mol^{-1}) Obtained in Gas Phase, Aqueous Solution, and the Enzyme Barriers for the Claisen and Cope Rearrangements in BsCM

	chorismate	carbachorismate
$\Delta G^{\ddagger}_g - \Delta G^{\ddagger}_w$	14.2	14.2
$\Delta G^{\ddagger}_g - \Delta G^{\ddagger}_e$	22.8	19.8
$\Delta G^{\ddagger}_w - \Delta G^{\ddagger}_e$	8.6	5.6

carbachorismate rearrangements in aqueous solution and BsCM are given in Table 2. From the geometrical point of view, these transition structures keep the same features rather than in gas-phase calculations: a concerted process but with somewhat smaller distances than in gas phase. However, from the energetic point of view, large differences are found.

In aqueous solution, the AM1/TIP3P activation free energies for the chorismate and carbachorismate rearrangements are 37.9 and 48.6 kcal/mol, respectively. By comparison of the AM1 and B3LYP/6-31G* free energy barriers in gas phase, we can expect an error of about 11.0 and 6.9 kcal/mol for the semiempirical Hamiltonian for the Claisen and Cope reactions. If we take into account this error, we can estimate the activation free energies ($\Delta G^{\ddagger}_{corr}$) to be about 26.9 and 41.7 kcal/mol for the Claisen and Cope processes in solution. Thus, the activation free energies are 14.2 kcal/mol lower in solution than those in gas phase for both reactions; see Table 3. This means that, within the computational errors, water solvation has the same catalytic effect for both processes. We attribute this energy barrier diminution upon solvation to the fact that the two negatively charged carboxylate groups could be now approached more easily than in gas phase since the electrostatic repulsion is severely reduced. The averaged distance between the two carboxylate groups (measured from carbon to carbon) diminishes from approximately 6 Å in the reactants to about 5 Å in the transition structures for both cases. This would mean a classical electrostatic contribution to the energy barrier of about 10 kcal/mol. Of course, the chorismate molecule can also form hydrogen bonds through the ether oxygen atom (O7), while the methylene group of carbachorismate cannot. In fact, the averaged atomic charge of O7 is slightly increased, in absolute value, when passing from the reactant state (−0.26 au) to the transition state (−0.33 au) in water, similarly to other Claisen rearrangements.[41] However, it seems that these additional interactions do not contribute in a decisive way to reduce the free energy barrier or are compensated, in the case of carbachorismate, by a more effective screening of the intramolecular electrostatic repulsion.

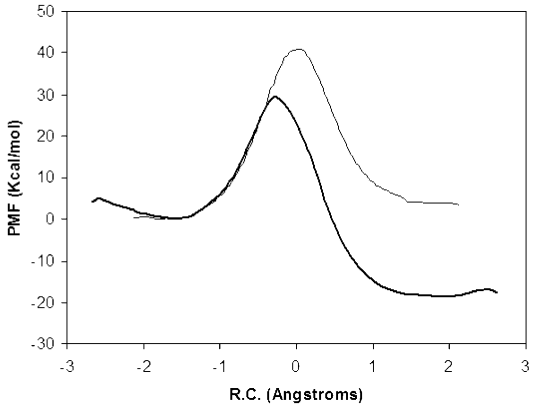

Figure 1. Potentials of mean force for the Claisen (bold line) and Cope (normal line) rearrangements in BsCM.

Potentials of mean force for the Claisen and Cope enzymatic reactions are presented in Figure 1. Using the hybrid AM1/OPLS calculations, the activation free energies for the chorismate to prephenate and carbachorismate to carbaprephenate rearrangements in BsCM are estimated to be 29.3 and 43.0 kcal/mol, respectively. If we take into account the error due to the semiempirical treatment of the quantum subsystem, our best estimation of the activation free energy ($\Delta G^{\ddagger}_{corr}$) for the Claisen and Cope rearrangements in BsCM would be 18.3 and 36.1 kcal/mol, respectively (Table 2). The former value is in reasonable agreement with the experimental data (15.4 kcal/mol).[42] These results mean that the enzyme diminishes the energy barriers for both rearrangements, chorismate to prephenate and carbachorismate to carbaprephenate. However, in contrast to the water solution, the enzyme is more efficient catalyzing the Claisen process. The free energy barrier for the Cope rearrangement in the active site of BsCM is reduced by 19.8 kcal/mol (at the AM1 level) with respect to the gas-phase value, while for the Claisen process the reduction amounts up to 22.8 kcal/mol. With respect to the aqueous solution processes, the enzyme diminishes the free energy barriers by 8.6 and 5.6 kcal/mol for the Claisen and Cope reactions, respectively (see Table 3). Thus, BsCM is more efficient catalyzing the Claisen reaction than the Cope one by 3.0 kcal/mol. The difference in the free energy barriers of both rearrangements is then larger in the enzyme active site than in the gas phase or in aqueous solution.

To analyze this specific effect of BsCM on the Claisen reaction, we can express the enzymatic free energy barrier ($\Delta G^{\ddagger}_e$) using a simple thermodynamic cycle as

$$\Delta G^{\ddagger}_e = \Delta G^{\ddagger}_w + [\Delta G^{TS}_{Bind} - \Delta G^{R}_{Bind}] \qquad (1)$$

where $\Delta G^{\ddagger}_w$ is the activation free energy in aqueous solution and ΔG^{X}_{Bind} stands for the binding free energy of the X state (reactants or transition state) from the aqueous solution. The last two terms in the right-hand side of eq 1 (ΔG^{TS}_{Bind} and ΔG^{R}_{Bind}) are negative by as far as the free energy barrier is lower in the enzyme than in solution $|\Delta G^{TS}_{Bind}| > |\Delta G^{R}_{Bind}|$. Using eq 1 for Claisen (O) and Cope (C) rearrangements and taking into account that the difference in the enzyme free energy

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barriers ($\Delta G^{\ddagger}_{e,O} - \Delta G^{\ddagger}_{e,C}$) is larger than in solution ($\Delta G^{\ddagger}_{w,O} - \Delta G^{\ddagger}_{w,C}$), we obtain the following inequality:

$$|\Delta G^{TS}_{Bind,O} - \Delta G^{R}_{Bind,O}| > |\Delta G^{TS}_{Bind,C} - \Delta G^{R}_{Bind,C}| \qquad (2)$$

That is, the preferential binding of the transition state with respect to the reactant state is larger for chorismate than for carbachorismate. We have then two different limiting cases as possible origins of the larger catalytic activity of BsCM for the chorismate rearrangement over the carbachorismate one: (i) enhanced interactions in the Claisen transition state relative to the Cope one ($|\Delta G^{TS}_{Bind,O}| > |\Delta G^{TS}_{Bind,C}|$) or (ii) in the Cope reactant state relative to the Claisen one ($|\Delta G^{R}_{Bind,C}| > |\Delta G^{R}_{Bind,O}|$). The real situation may be intermediate between these extremes.

We have studied these two possible sources of catalysis from a geometrical point of view, analyzing the substrate−enzyme interactions with the transition and the reactant states and also the preorganization exerted by the enzyme in both reactants. According to our previous analysis, the larger catalytic effect of BsCM on the Claisen reaction should be reflected in stronger interactions with the corresponding TS and/or a larger preorganization effect on the chorismate molecule.

Enzyme−Substrate Interactions. We have compared the specific substrate/active site interactions for the reactants and transition states of the Claisen and Cope processes. Figure 2 displays some key residues of the active site with the transition structures of both reactions. This picture shows the interaction pattern established between the substrate molecule and the enzyme residues. With respect to the enzyme structure, it must be noticed that Arg90 suffers an important displacement when chorismate is substituted by carbachorismate, while the rest of the active site residues remain at very similar positions.

Figure 3 shows selected averaged distances between substrate and enzyme atoms for the reactants and transition states of the Claisen and Cope rearrangements. It is interesting to note that, for both reactions, carboxylate oxygen atoms (O16/O17 for the ring carboxylate group and O12/O13 for the side chain one) establish strong interactions with arginine residues (Arg63, Arg116, Arg90, and Arg7). As it has been shown,[43] the ring carboxylate group establishes less strong interactions with the protein than the side chain one. These interactions can play an important role not only during the substrate binding but also during the progress of the chemical rearrangement. Thus, while some of these Arg−carboxylate interactions are weakened when passing from reactants to transition states (see the averaged distances between O16/17 and with HH11 of Arg116 and O12/13 with HH22 of Arg7 and HH21 of Arg90), others are strengthened (O12/13 with HH11 of Arg7 and with HE of Arg90). These results suggest that the arginine network in the active site would play an active role allowing the carboxylate groups to evolve according to the reaction progress. In the same direction, Tyr108 establishes a strong interaction with the carboxylate group of the side chain (O12/O13) only in the transition state, and thus it would contribute to diminishing the energy barrier. The effect of this particular residue is discussed in more detail below. As a consequence of the hydrogen bond network established in the BsCM active site, the two negatively charged carboxylate groups can be approached more easily than in gas phase. The intramolecular electrostatic repulsion is compensated by means of substrate−enzyme interactions, resulting in an important diminution of the energy barrier for both reactions. Apart form the carboxylate oxygens, the hydroxyl oxygen (O8) shows strengthened interactions with HG1 of Cys75 when passing from reactant to transition states for both reactions. Interestingly, it has been proposed that interactions between this group and the enzyme may contribute significantly to rate acceleration in the case of *E. coli* chorismate mutase.[44] Although it cannot be deduced only from a distance analysis, the global balance of electrostatic interactions between transition and reactant states is favorable to the former. We recently estimated that the averaged electrostatic stabilization of the Claisen transition state relative to the ground state in BsCM amounts up to 17.1 kcal/mol.[9] This value is not a proper free energy because it was obtained as a difference between internal energies. More recently, analyzing particular reaction paths, Mullholand et al.[45] found that the stabilization provided by the enzyme is maximum at the transition structure. Warshel et al.[46]

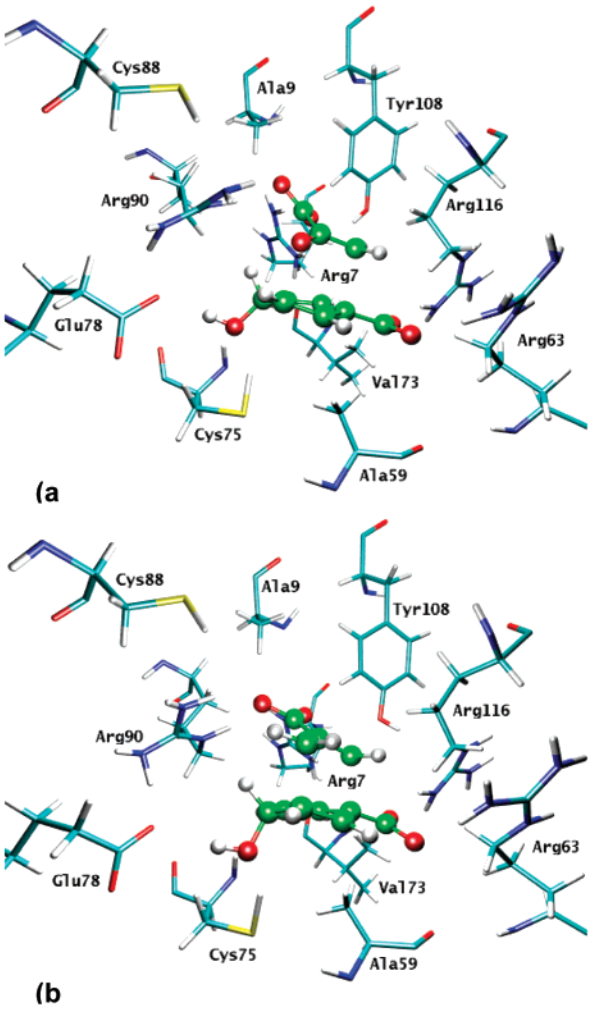

Figure 2. Details of the transition structures obtained for the (a) chorismate to prephenate rearrangement and (b) carbachorismate to carbaprephenate rearrangement in the active site of BsCM. Some residues have not been presented for clarity purposes.

(43) Mandal, A.; Hilvert, D. *J. Am. Chem. Soc.* **2003**, *125*, 5598−5599.

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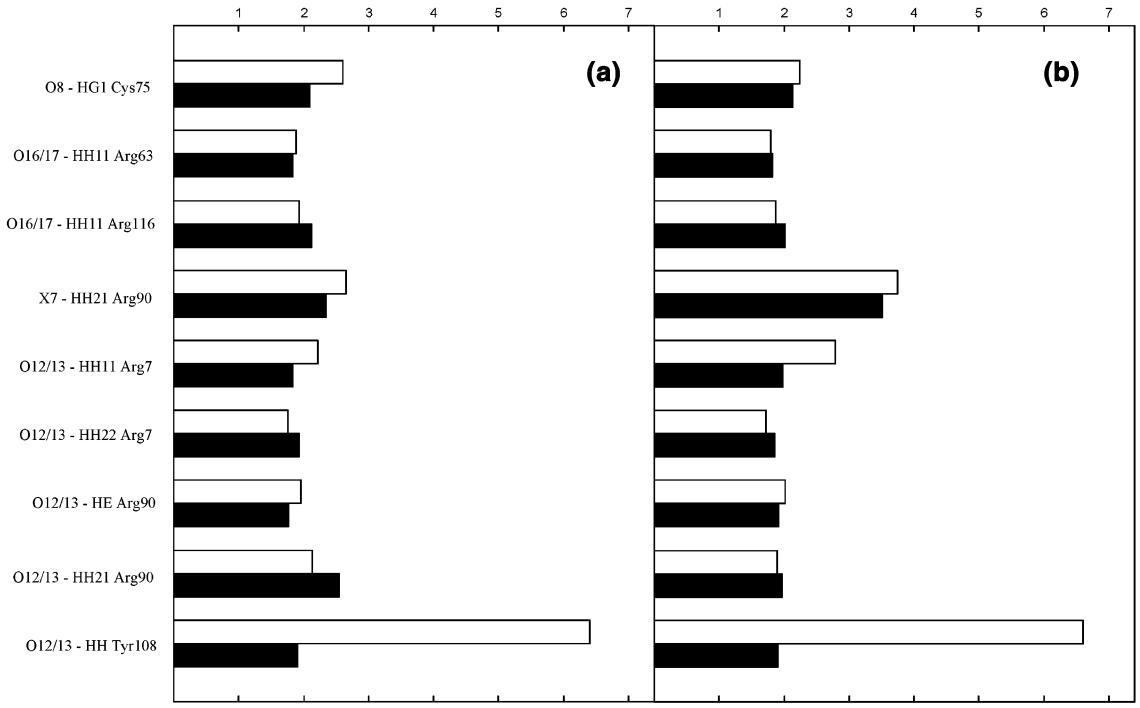

Figure 3. Selected interatomic averaged distances between the substrate and some of the residues of the enzyme for reactants (white bars) and TS (black bars): (a) chorismate to prephenate rearrangement and (b) carbachorismate to carbaprephenate rearrangement.

arrived to the same conclusion using linear response approximation calculations.

Our structural analysis of chorismate transition state interactions in the active site is in good agreement with the interactions reported for an endo-oxabicyclic transition state analogue.[47] We can also compare our structural analysis with the results of mutagenesis studies of active site residues in BsCM.[48] According to these studies, Tyr108, Arg116, and Cys75 appear to contribute similarly to the binding energy of substrate and transition state. Our findings seem to agree in the case of Arg116 but not for Ty108 and Cys75, for which we found enhanced interactions with the transition state. The role played by Tyr108 deserves some additional comments. In fact, we found direct hydrogen bonding between Tyr108 and the carboxylate group of the side chain (O12/O13) in some minimized structures belonging to the reactant state,[7] while Mullholand et al.[45] localized a transition structure in which this hydrogen bond was lost. In our molecular dynamic simulations of the reactant state, we have observed that, starting from slightly different initial conditions, the Tyr108 hydroxyl group can establish a hydrogen bond either with the side chain carboxylate group of the substrate or with a water molecule (as found in ref 45) or even with other neighboring residues. Thus, it must be taken into account that an analysis based only in distances may be insufficient to quantify the effect of a particular residue on the binding energies and, on the other hand, mutagenesis of a particular residue can imply more changes than expected in the structure of the active site.

The previous analysis would explain the reduction of the free energy barriers in both processes. To clarify the origin of the extra energy barrier reduction in the Claisen rearrangement with respect to the Cope one we can compare the interactions established between reactants and transition states of the two processes. Obviously, there is an important difference when comparing the Claisen and the Cope transition states: in the former process, the ether oxygen (O7) presents an interaction with Arg90 that it is reinforced when passing from the reactant to the transition state. The averaged Mulliken charge of this atom in the enzymatic environment increases, in absolute value, from -0.25 to -0.36 au when passing from reactant to transition state, and simultaneously the averaged distance to HH21 of Arg90 is reduced, increasing the electrostatic interaction. In agreement with this, in the aforementioned mutagenesis study,[48] it was found that Arg90 stabilizes the transition state charge distribution. The key role played by this specific interaction was already pointed out in the structural analysis of Lipscomb et al.,[47] and it has been also recently evidenced by means of the substitution of this residue by citrulline, an isosteric but neutral Arginine analogue.[49] Finally, Mullholand et al.[45] found that the maximum contribution of individual residues to the electrostatic stabilization of the transition structure relative to the reactants was provided by Arg90 (about 3−4 kcal mol^{-1}). Of course, this hydrogen bond is not present in the Cope rearrangement as far as the oxygen atom is substituted by an apolar CH_2 group (with an averaged Mulliken charge of 0.11 au in the transition state). This interaction would then be responsible, at least partially, for the enhanced catalytic activity of BsCM with chorismate rearrangement as compared to carbachorismate by means of an overstabilization of the Claisen transition state with respect to the Cope one ($|\Delta G^{TS}_{Bind,O}| > |\Delta G^{TS}_{Bind,C}|$). The difference with the reaction in water is that in the enzyme the hydrogen bond is established with a positively charged residue, resulting in a stronger interaction as reflected by the larger polarization of O7 in the enzyme than in water.

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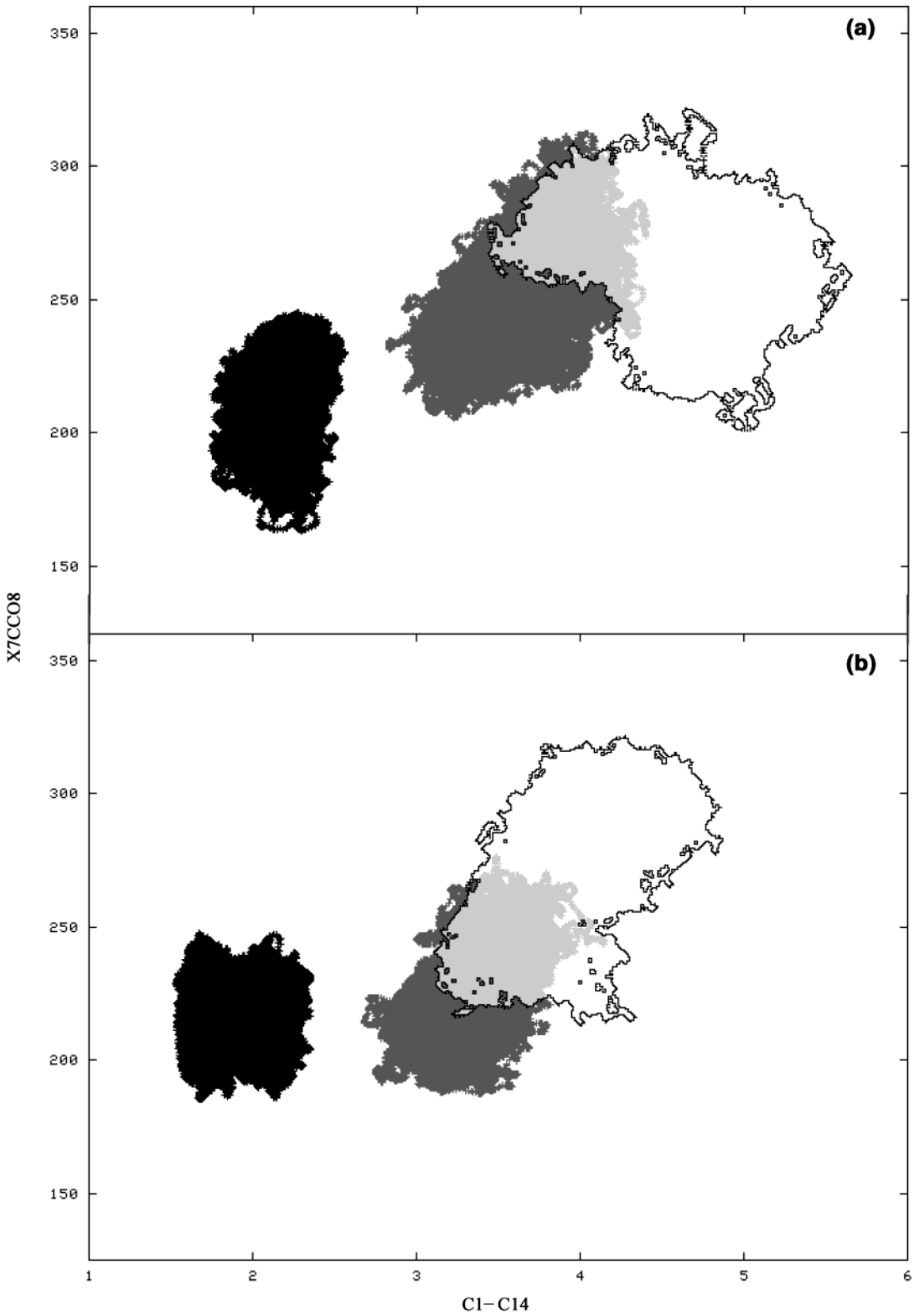

Figure 4. QM/MM trajectories (C1−C14 distance in Å and X7CCO8 dihedral angle in deg) corresponding to reactant conformers in aqueous solution (white), reactant conformers in the enzyme (gray), and transition structures (black) of the (a) chorismate to prephenate rearrangement and (b) carbachorismate to carbaprephenate rearrangement. Superposition of reactants trajectories obtained in both media is displayed in light gray.

Substrate Preorganization. Once analyzed we the specific electrostatic interactions established in the BsCM active site, we also investigated the ability of this enzyme to constrain the substrate molecule in a reactive conformation for both reactions, here called the substrate preorganization effect. This should not be confused with the enzyme preorganization concept introduced by Warshel et al. to describe the reduction of the enzyme reorganization energy.[50] A consensus has still not been reached about the terminology, and this substrate preorganization effect is also termed in the literature as the steric restraint effect[17a] or

(50) (a) Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5250−5254. (b) Warshel, A. *J. Biol. Chem.* **1998**, *273*, 27035−27038.

near attack conformation (NAC) effect.[15] As pointed out above, destabilization of the Claisen reactant state with respect to the Cope one could be also invoked to explain the differential catalytic activity of BsCM on both reactions.

We recently showed that chorismate conformers may be classified according to two different criteria: the distance between the carbon atoms to be bonded (the C1−C14 distance) and the pseudodiaxial/pseudodiequatorial disposition of the ring substituents (the X7C5C4O8 dihedral angle).[4,9] Obviously, the chorismate reactive conformer is the most similar to the transition structure: the one having smaller C1−C14 distances and a pseudodiaxial disposition of the hydroxyl and the ether bridge (smaller X7C5C4O8 dihedral angles). In gas phase, the most stable chorismate conformers are the pseudodiequatorial forms, because they are the only ones able to establish an intramolecular hydrogen bond between the hydroxyl and the side chain carboxylate group.[4] In aqueous solution, the pseudodiequatorial/short distance conformer is the most stable one.[3,4,9] In the enzyme, if the active site interactions with the substrate carboxylate groups allow for an easier approach of these groups during the reaction, they must also favor those reactant conformations where these groups are closer. In other words, the enzyme favors those reactant conformations which are more similar to the transition structure: the pseudodiaxial/short distance form. To analyze this point for carbachorismate in comparison with chorismate, we have carried out molecular dynamics simulations of the enzymatic transition states and the reactants both in the enzyme active site and in aqueous solution. The distribution of structures found during the simulations is shown in Figure 4a and b for the Claisen and Cope rearrangements, respectively. For both reactions, the trajectories of the corresponding transition states show a similar behavior with a large overlap region. Transition structures have a clear pseudodiaxial/short distance character, with slightly larger averaged distances (see Table 2) and dihedral angles (220° versus 218°) in the case of the Claisen reaction. The preorganization effect exerted by the enzyme on the reactant structure is evidenced by comparing the dynamics of the reactants in aqueous solution and in the enzyme active site. For both the Claisen and the Cope reaction, the trajectories of the reactants in the enzyme are closer to the transition states than in aqueous solution: the reactant structures in the active site present shorter carbon−carbon distances and a more pseudodiaxial character (smaller dihedral angle) than in aqueous solution. For chorismate, the effect of the enzyme is essentially on the C1−C14 distance, which is shortened (in averaged values) from 4.7 Å in aqueous solution to 3.6 Å in the active site, while the O7C5C4O8 dihedral angle is only slightly reduced (from 266° to 251°). For carbachorismate, we have a smaller reduction of the C1−C14 distance (from 4.0 to 3.3 Å) but a larger effect on the dihedral angle (from 277° to 220°). To optimize the interactions between the side chain carboxylate group (O12/O13 oxygens) and the active site arginine residues, the carbachorimate structure is more deformed than chorismate, with respect to the in-solution dynamics. The reason is found in the displacement observed for Arg90 in Figure 2. When O7 is substituted by a methylene group, the O7−Arg90 hydrogen bond interaction disappears and this residue can move inside the active site pocket. In this way Arg90 establishes now stronger interactions with the O12/O13 oxygen atoms (the distances between Arg90 and this carboxylate group are slightly shorter for the carbachorismate analogue; see Figure 3). These enhanced interactions force a larger displacement of the carboxylate group. The final results are that the X7C5C4O8 dihedral angle is smaller in carbachorismate than in chorismate, where the Arg90 residue is displaced outward from the active site, interacting with the ether oxygen atom.

From the analysis of the reactants trajectories, it is evident that BsCM restrains the carbachorismate molecule in a conformation close to its corresponding transition state more than it does with chorismate. That is, the preorganization effect is larger for the Cope reaction. It is also interesting to compare these results with the predictions based in the near attack conformation (NAC) concept,[15] recently applied to analyze the effect of *E. coli* chorismate mutase.[10,18] According to this, the main enzymatic effect is to favor some particular fluctuations of the reactant's arrangements closer to the transition structure. This would lead to a reduction in the activation free energy. However, what we have found is that the catalytic activity of BsCM is lower for carbachorismate than for chorismate by 3 kcal mol^{-1}. So, to explain the larger catalytic effect found on the Claisen process, we need to invoke the preferential stabilization of the Claisen transition state by means of specific interactions in the active site. Obviously, this comparative analysis does not exclude the existence of a preorganization or NAC effect favoring the Cope reaction, but the results here presented suggest that the dominant effect is the electrostatic interactions on the transition state. The electrostatic stabilization of the Claisen transition state could be larger than 3 kcal mol^{-1}, being the overall effect slightly reduced by the preorganization of the Cope reactants.

4. Conclusions

We have presented a detailed QM/MM and statistical analysis of the Claisen and Cope rearrangements of chorismate and carbachorismate catalyzed by BsCM. These two reactions are described as concerted processes that present very different intrinsic or gas-phase free energy barriers, being about 10−15 kcal mol^{-1} larger for the Cope reaction. The electrostatic interaction pattern established in aqueous solution and in the enzyme active site with carboxylate and hydroxyl groups of the substrate considerably reduces the calculated free energy barriers in both cases. However, the ability of the enzyme to reduce the energy barrier is larger (by about 3 kcal mol^{-1}) in the case of the Claisen reaction. This differential catalytic effect is mainly attributed to the enhanced electrostatic interaction established between O7 and Arg90 in the transition state of the chorismate to prephenate rearrangement.

The electrostatic interactions established between the substrate and the enzyme play a primary effect reducing the free energy barriers, but in addition, they also have consequences on the reactant structures. If the transition structures are going to be stabilized, then those reactant structures closer to these would be also relatively favored. The BsCM active site displays a similar effect on chorismate and carbachorismate molecules. In the active site, reactant structures have smaller carbon−carbon distances and more pseudodiaxial character than in solution, becoming then more similar to the transition structures. The resulting averaged reactant structure of carbachorismate is closer to the transition structure than in the case of chorismate. The reason is found in the displacement of Arg90 toward the ether

bridge carboxylate group when the hydrogen bond interaction with ether oxygen is lost.

The main conclusion is that the electrostatic stabilization of the transition state is the dominant effect to explain the larger catalytic effect of BsCM on the Claisen reaction, although a larger preorganization of the Cope reactant should be also considered for the global energetic balance. This is in agreement with the analysis provided by Hilvert et al.[21] Comparing the effect of the same enzyme (BsCM) on two different substrates (chorismate and carbachorismate), we have been able to obtain conclusions about the relative magnitude of the preorganization and transition state stabilization effects. The procedure employed here can be useful to highlight the origin of enzyme catalysis by comparing not only a unique enzyme with different substrates but also a unique substrate with different enzymes (for example, mutants).

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