Catalytic Antibodies

Towards a Rational Design of Antibody Catalysts through Computational Chemistry**

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Pauling's seminal idea concerning enzymatic catalysis is that an enzyme lowers the energy of the transition state (TS).[1] Evidence in support of this proposal is the fact that stable

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compounds that resemble the TS, transition state analogues (TSAs), are competitive inhibitors of enzymes.[2] One approach to TSA design is to establish the nature of the enzymatic TS and to synthesize chemically stable analogues with similar features.[3] Raso and Stollar pioneered the use of TSAs as haptens in immunization processes to synthesize new catalysts: catalytic antibodies (CAs).[4] The study of processes associated with the activity of CAs provides opportunity to examine and understand enzyme catalysis and vice versa. In-depth knowledge of enzyme activity can be used to improve the specificity, selectivity, and efficiency of these new catalysts.[5] CAs are especially interesting as catalysts for those reactions for which no enzyme is known.[6]

The simplest kinetic scheme used to understand enzymatic and CA processes is that proposed by Michaelis and Menten, which proceeds with the formation of a substrate–catalyst complex (the Michaelis complex, MC) before the product-forming step during which the catalyst is recovered.[7] In the simplest version the reaction takes place through a single TS [Eq. (1)].

$$E + S \rightleftharpoons MC \rightarrow TS \rightarrow P + E \quad (1)$$

The activation free energy of the catalyzed reaction step can be related to that of a counterpart uncatalyzed process through the binding energies of the MC and the TS [Eq. (2)].

$$\Delta G^{\ddagger}_{cat} = \Delta G^{\ddagger}_{uncat} + \Delta G^{TS}_{bind} - \Delta G^{MC}_{bind} \quad (2)$$

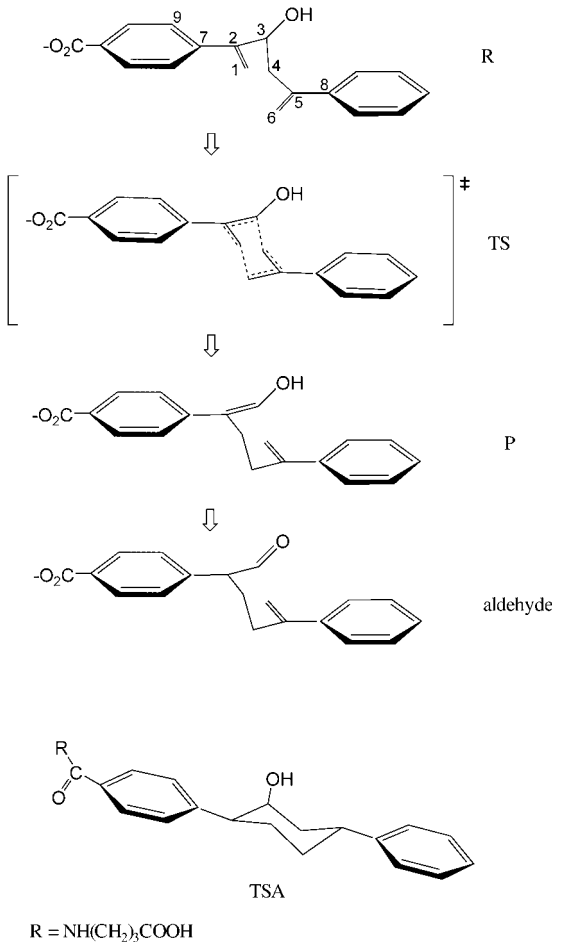
Here $\Delta G^{\ddagger}_{cat}$ and $\Delta G^{\ddagger}_{uncat}$ are the free energies of activation for the catalyzed and the uncatalyzed reactions, while ΔG^{TS}_{bind} and ΔG^{MC}_{bind} reflect the affinities of the protein for the TS and the MC, respectively. According to this scheme, the catalytic power of enzymes comes from the larger affinity of the enzyme for the TS than for the MC, since $\Delta G^{TS}_{bind} - \Delta G^{MC}_{bind}$ is a negative quantity. Antibodies are synthesized on the basis of their affinity for a TSA (ΔG^{TSA}_{bind}), a quantity expected to be correlated to the binding energy of the true TS of the reaction to be catalyzed (ΔG^{TS}_{bind}). Thus, CAs are expected to provide a lower activation free energy.

However, initial expectations for CAs as catalysts have not been fully met. First of all, CAs are not as efficient as the enzymes. Second, not all antibodies that stabilize TSAs are catalysts of the reaction. Finally, there are cases where after a process of maturation (which results in an increased affinity of the CA for the TSA) a paradoxical decrease in the catalytic power is observed, relative to the initial or germline CA.[8]

Different arguments have been proposed to explain these findings. The fact that CAs present modest rate enhancements relative to those of enzymes could be due to the low affinity between the CA and the TSA that can be developed by the immune system. This affinity is apparently not enough to result in the TS affinity required for a substantial increase of the reaction rate. During the process of maturation, the TSA–CA affinity (in terms of the dissociation constants calculated for the TSA) increases to ca. 10^{-10} while the TS–enzyme affinity increases to 10^{-23}.[7] Secondly, even when the CA–TSA affinity could be improved enough, to date it has been difficult to elicit antibodies that are as effective at differentiating the ground state from the TS.[7] In this regard, Schultz et al. have stressed the lack of flexibility of the catalytic antibodies.[9] The introduction of somatic mutations, which lead to an increase in binding affinities, can cause a significant restriction in the relative orientation of the substrate, thereby decreasing the rate constant.[9] The increased binding affinity of the affinity-matured antibody stabilizes the substrate in a catalytically unfavorable conformation. Thus, it would be possible to rationalize why some CAs do not act as catalysts. Finally, as also suggested by Bartlett and Mader,[7] it is not possible to devise very accurate TSAs; that is, the TSA cannot be similar enough to the true TS. As a consequence, an improvement in ΔG^{TSA}_{bind} would not be directly translated into an improvement in ΔG^{TS}_{bind}. All these problems arise because of an important issue of timescales. In its continuous processes of evolution, nature has brought forth countless mechanisms for complex biochemical reactions, but the biological selection process that produces the antibody differs from that governing enzyme evolution by an enormous factor: a timescale of weeks in the former in contrast to a process occurring over millions of years in the latter.

The methods and techniques of computational chemistry provide excellent tools for obtaining molecular details of catalytic processes.[10–13] In this paper we use these tools to show that developing a good catalyst requires consideration of both the TS and the MC. With the aim of increasing the stability of the TSs, scientists design CAs based on their affinity to TSAs. In this process the effect of the CA on the reactant state is completely lost. We suggest that CAs usually present low catalytic efficiency (relative to that of enzymes) and even inverse correlations between maturation process and catalytic power. This can be explained not only because the TSA does not properly represent the TS, but mainly because the MC is not considered in the improvement process. Enzymes and CAs have evolved with different purposes: the former to decrease the activation free energy of the reaction, and the latter to increase the binding energy for the TSA (and for the TS). In the first case the target is the difference between the binding energy of the TS and the MC, ($\Delta G^{TS}_{bind} - \Delta G^{MC}_{bind}$), while in the second case attention is focused on ΔG^{TSA}_{bind} (which is related to ΔG^{TS}_{bind}), and the MC is not considered at all.

CAs have been produced for a plethora of chemical reactions including hydrolytic reactions, sigmatropic rearrangements, and cycloadditions, carbon–carbon bond-forming reactions, and redox reactions.[14] In this study we have selected an oxy-Cope rearrangement catalyzed by AZ-28 and related antibodies.[15] Apart from the advantages of this system from the computational point of view,[16] no enzyme is known for this reaction. The monoclonal AZ-28 catalyzes the unimolecular rearrangement of a substituted hexadiene to the corresponding aldehyde (see Scheme 1). The immune system provides a large number of different germline antibodies as an initial response to a foreign molecular structure. In this process the antigen is the TSA, depicted in Scheme 1. Once a germline antibody is selected from the pool based on its affinity for an antigen, an additional process of affinity maturation follows, in which random somatic mutations are



Scheme 1. Schematic diagram of the molecular mechanism of the oxy-Cope reaction and detail of the TSA.

selected to obtain a high-affinity antibody. In the case of the AZ-28, it was found that the matured AZ-28 presented a 40-fold higher affinity for the TSA but afforded a 30-fold lower rate enhancement than its germline precursor.[8] Analysis of the effects of individual amino acid mutations indicated that the SerL34Asn substitution was largely responsible for the decrease in catalytic efficiency during affinity maturation.[8] We can now explain why the affinity-matured antibody is less efficient than its precursor germline antibody.

The mechanism of catalysis of the antibody-catalyzed oxy-Cope rearrangement has been theoretically study by Kollman et al.[17] using ab initio molecular orbital calculations and classical molecular dynamics simulations, and recently by Houk et al.[18] by means of gas-phase density functional theory calculations and flexible docking. In this paper we describe the application of a hybrid quantum mechanics/molecular mechanics (QM/MM) methodology already tested in our group on similar enzymatic pericyclic reactions.[19] As a first result of our study, free energy profiles for the AZ-28 catalytic antibody in its germline and matured form are plotted in Figure 1. The profiles indicate an associative mechanism described by two TSs; the first and rate-limiting step is formation of the C1–C6 bond, and the second step is breaking

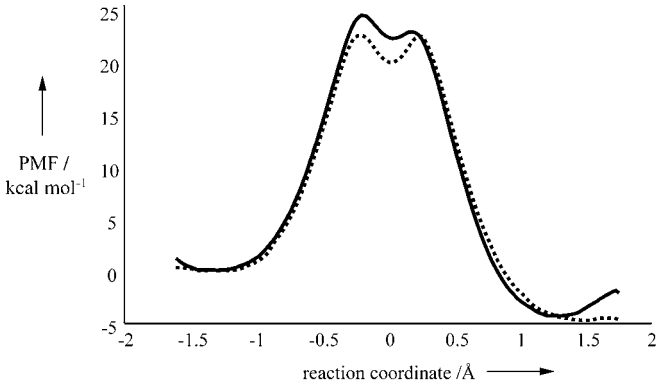


Figure 1. Free energy profiles in terms of the potential mean fore (PMF) for the AZ-28 catalytic antibody in its germline (dotted line) and matured forms (solid line) for the R-to-P transformation (see Scheme 1). The reaction coordinate is the antisymmetric combination of the interatomic distances of the forming and breaking bonds, C1···C6 and C3···C4, respectively. For details see ref. [19].

of the C3–C4 bond.[20] The oxy-Cope rearrangement catalyzed by the matured AZ-28 involves a slightly higher barrier than that catalyzed by its germline counterpart. Our calculations render a difference in the free energy barriers of 1.9 kcal mol^{-1}, which can be directly compared with the experimental result of 2.2 kcal mol^{-1}, obtained by Schultz et al.[8] This agreement allows us to be confident in our calculations and consequently to analyze the origin of this difference in free energy barriers by means of theoretical simulations.

Molecular simulations provide us with a detailed knowledge of chemical processes in terms of structures and energies. In Figure 2 we present some snapshots representative of the averaged structures of TSA, TS, and MC obtained from the QM/MM MD trajectories of the oxy-Cope rearrangement in the active sites of the germline and matured antibodies.[21] Some key residues and interactions are also depicted. The first conclusion that can be extracted from the analysis of the structures is that the cavity in the matured antibody is narrower than that in its germline counterpart. The compression between the light and heavy chains can be measured by the distance between the carboxylate OD1 or OD2 oxygen atoms of H101 and terminal hydrogen atom of L34 residues (HG1 for Ser or HD21 for Asn), located in the outer part of the catalytic cavity. Thus, the germline antibody presents a distance of about 4.3 Å, while in the matured antibody this distance is significantly shorter, 2.2 Å. Therefore, it is evident that the germline CA has a larger cavity than the matured CA for accommodating the TS and the MC, in accordance with Schultz's experimental results.[9]

Following with the analysis of Figure 2, the observed pattern of interactions in the CA–substrate complex reveals important differences. Starting from the TSAs, the hydroxy substituent of the cyclohexyl ring is hydrogen-bonded to Glu35 in both CAs (2.25 and 1.98 Å for the germline and the matured antibodies, respectively). Other TSA–CA interactions are also shorter for the matured antibody than for the germline counterpart, reflecting improved matching with the

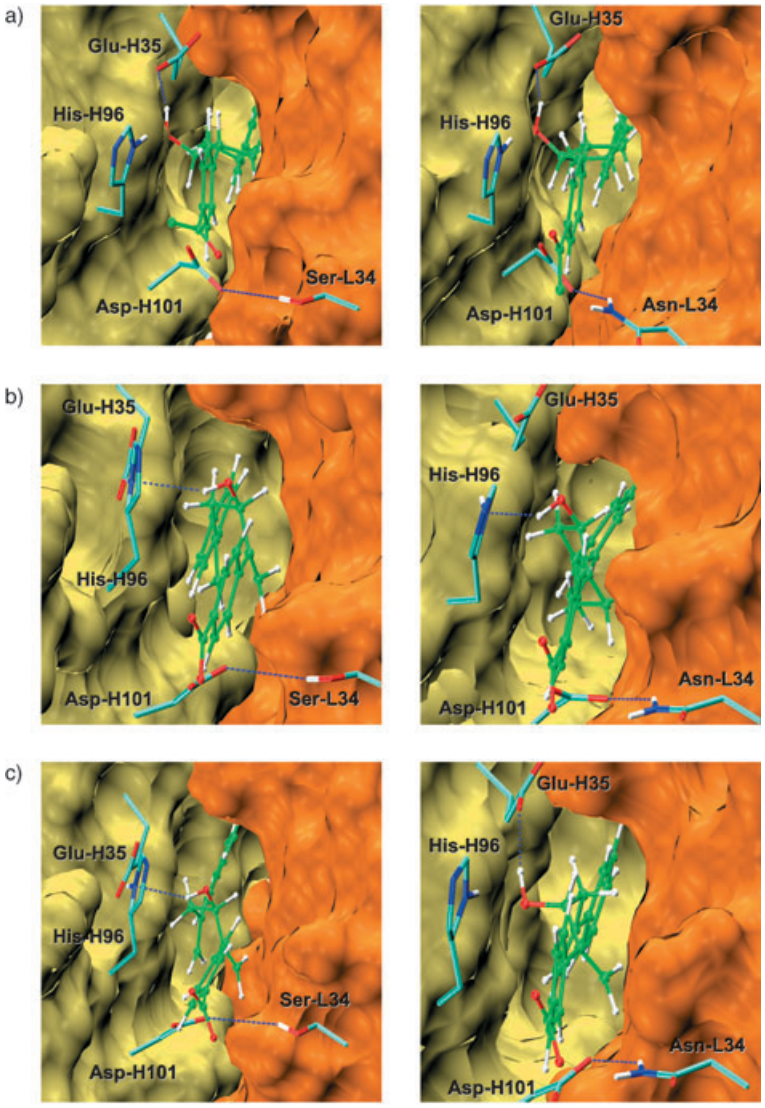


Figure 2. Detail of the averaged structures of the a) TSA, b) TS, and c) MC of the oxy-Cope rearrangement in the active site of the germline (left) and matured AZ28 antibodies (right). The heavy and light chains are shown in yellow and orange, respectively.

substrate after the maturation process. Another observed feature is the relative position of the cyclohexane ring (which is always in a chairlike conformation) with respect to the two aryl substituents. The observation that the cyclohexane ring is almost perpendicular to the substituents is in agreement with X-ray diffraction analysis.[9] Nevertheless, an important difference with the X-ray structure is the position of substrate in the antibody cavity. During our MD simulations the substrate moves inside the cavity, changing the initial hydrogen bond between the hydroxy group and the Glu50 through a bridging water molecule into a new interaction with the Glu35 located on the inside of the cavity. Packing effects in the X-ray diffraction structures could account for this difference.

In the case of TS–CA complexes, the first observation is the different orientation of the hydroxy substituent with respect to that in the TSA–CA complexes. The hydroxy group is now hydrogen-bonded to the His96 (2.84 and 2.79 Å for the germline and the matured antibodies, respectively). The dramatic change in the pattern of interactions on going from the TSA to the TS is due to the different hybridization of the C2 and C5 atoms. These carbon atoms present sp^2 and sp^3 hybridizations in TSs and TSAs, respectively, defining an important change in the orientation of the reactive ring. As a result the hydroxy substituent cannot interact with the same residues in the TS and the TSA, as commented above. The new interaction is established by means of rotation around the C2–C3 bond. As previously postulated in the literature, the hydrogen bond between the hydroxy group and His96 may enhance the rate of the process by increasing the electron density on the hydroxy oxygen atom.[14,22] In order to estimate quantitatively this prediction, Baumann et al.[22] and Haeffner et al.[23] have calculated the effect of some substituents on the rates and mechanisms of oxy-Cope rearrangements by means of simple molecular models in the gas phase with a density functional theory based method. These results confirm that the greater the electron density on the hydroxy group, the lower the barrier height. From these observations it is obvious that the use of a TSA that better resembles the TS would be the first step to designing a more efficient CA. Some strategies that work in this sense have already been postulated,[24,25] but it is not the aim of this study.

Regarding the MC structures, it is amazing that while the pattern of interactions of the germline CA remains almost invariable with respect to its TS (2.71 Å for the hydrogen bond between the hydroxy group and His96), the orientation and interactions of the MC in the matured cavity change dramatically; the MC assumes a conformation close to that of the initial TSA (the hydroxy group is now hydrogen-bonded to Glu35, with an average distance of 2.16 Å, and not to His96). The shorter intermolecular distances between the matured CA and the substrate cause the MC to be trapped in a conformation resembling the TSA rather than the TS, thus making the reaction less favorable. This observation can be tested by the values of the reaction coordinate of the MCs (−1.56 for the matured and −1.47 for the germline antibodies, see Figure 1), as well as the distance C1–C6 of the forming bond (3.10 and 3.03 Å for the matured and germline, antibodies, respectively). In both cases, the germline CA presents values closer to the TS than the matured antibody does.

To gain deeper insight into the different behavior of the germline and matured CAs, we determined the energy of the interaction between the substrates and the antibodies (Table 1).[26] From the values listed, it can be observed firstly

Table 1: Interaction energy differences [kcal mol^{-1}] of matured and germline AZ-28 antibodies with different substrates.

Substrate	E_{int} (matured)	E_{int} (germline)	ΔE_{int}
TSA	−232.4	−228.3	−4.0
TS	−199.0	−193.6	−5.3
MC	−197.6	−190.2	−7.4

that TSA interacts better with the matured CAs than with the germline CAs, and secondly that the TSA species present larger interaction energies than the TSs and MCs. Both results are quite logical when one considers the nature of the affinity maturation process. From the mechanistic point of view, the comparison between the stabilization of the TS and the MC by the catalytic antibody on going from the germline to the matured forms allows us to conclude that the maturation process produces a larger effect in the MC (−7.4 kcal mol^{-1}) than in the TS (−5.3 kcal mol^{-1}). The difference between these values (2.1 kcal mol^{-1}) is almost equivalent to the differences in free energy barriers obtained both experimentally (2.2 kcal mol^{-1}) and theoretically (1.9 kcal mol^{-1}). Nevertheless, we must bear in mind that the estimated free energy barriers plotted in Figure 1 are only related to the MC-to-TS step of Equation (2). The fact that the equilibrium constant of the MC formation was proved experimentally to be almost equal for the germline and the mature CAs[8] is not in agreement with the different interaction energies given in Table 1 for the MC complex. This means that although entropic and other enthalpic terms make different contributions to the substrate binding energy in the germline and mature antibodies, the similarity between free energy barriers and the interaction energies suggests that the source of the different catalytic efficiencies may be attributed to the larger CA–MC interaction in the mature CA. The analysis of the interaction energy shows that this term is mainly electrostatic in nature.

From these results, it is clear that our methodology explains the difference in CA activity for the germline and matured antibodies. Combining the results of different mutations, it has been demonstrated experimentally that the particular AsnL34Ser mutation in the matured CA lowers the free energy barrier by 1.0 kcal mol^{-1}.[8] The effect of this mutation can be analyzed by means of our computational methodology by introducing it in the MC–CA complex of the matured antibody. Hybrid QM/MM molecular dynamics simulations started from different MC structures with the matured antibody, in which the Asn-L34 residue was changed to Ser, have shown a spontaneous opening of the cavity after 0.5–1 ns. This effect is accompanied by a change in the pattern of interactions, establishing the same pattern as that observed in the germline CA. In particular, the hydrogen bond between the hydroxy substituent and Glu35 is broken, and a new interaction is established with His96. The generation of a free energy profile for this new CA yields an activation free energy of 23.7 kcal mol^{-1}, which is 0.7 kcal mol^{-1} lower than that of the matured antibody and in good agreement with the decrease in the barrier observed by Schultz et al.[8] While the matured antibody displays an enhancement in the affinity for the TS, a single mutation can improve its catalytic power by changing the interaction with the MC. In this way, the target quantity in Equation (2), $\Delta G^{TS}_{bind} - \Delta G^{MC}_{bind}$, is optimized. The biological selection of enzymes by evolution can be imitated by antibodies.

It has always been the ambition of research chemists to imitate nature in its magnificent methods for generating complex chemical structures with corresponding chemical reactivity. The present work shows how computational techniques can be crucial to approaching nature's power in evolution. The rational design of a CA requires an in-depth knowledge of enzyme catalysis. First of all, the choice of an appropriate stable TSA, as close as possible to the TS of the reaction to be catalyzed, is crucial to obtaining an efficient CA. In addition, a favorable balance of interactions between the antibody and the relevant structures that the substrate adopts along the full chemical process: the MC and the TS. In improving the activity of a CA the interesting mutations are those that stabilize both the TS and the MC but, as a general feature for all catalysts, the stabilization of the MC must not be too strong. As pointed out in the Sabatier principle,[27] the interactions of the catalyst–substrate complex must be strong enough to favor its formation but not so strong that the catalyst becomes an inhibitor. The maturation process succeeds in stabilizing the TSA and to some extent the TS but not in controlling the relative stabilization of the TS with respect to the MC. We have shown in this paper that computational chemistry enables the study and prediction of which mutations can stabilize the TS relative to the MC, improving the design of the CAs. The combination of theoretical and experimental studies appears to be essential to improving the efficiency of new semisynthetic biological catalysts, thus imitating nature in enzyme evolution.

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[19] For a general review of the methodology we are currently applying to study enzyme reactions see ref. [28]. Based on the original idea of the hybrid quantum mechanical/molecular mechanical methods,[29] in this particular antibody-catalyzed oxy-Cope rearrangement, free energy profiles (in terms of potential of mean force) were obtained with the umbrella-sampling approach[30] implemented in the Dynamo program.[31] We sampled all the degrees of freedom but the distinguished reaction coordinate (the antisymmetric combination of the interatomic distances of the forming and breaking bonds, C1···C6 and C3···C4 as depicted in Scheme 1) by means of a series of molecular dynamic simulations. Umbrella sampling was used to place the system at an adequate value of the reaction coordinate (ζ_0) and then molecular dynamics simulations were run. The fluctuations of the reaction coordinate are finally pieced together by means of the weighted histogram analysis method (WHAM)[30] obtaining the full distribution function and thus the free energy profile. The system consisted of a cubic box with sides of 79.5 Å in which the CA and 15492 water molecules (50167 atoms in total) were all treated using the OPLS force field,[32] plus the substrate (39 atoms) which was was treated by AM1 semiempirical Hamiltonian. Periodic boundary conditions were used in all calculations. Because of its size, only those CA atoms within 30 Å of the substrate center of mass were allowed to move (~11500 atoms, model dependent), and the positions of the remaining atoms were frozen.
[20] We have also explored the potential energy surface of the reaction by means of gas-phase calculations with density functional theory methods (B3LYP/6-31G*) as implemented in the Gaussian03 package of programs.[33] Our results render two mechanisms, depending on the orientation of the hydroxy substituent (inwards or outwards the cyclohexane ring). Both mechanisms involve a cyclohexanediyl intermediate in one step. Gas-phase AM1 calculations have been also carried out in order to test the suitability of the semiempirical method to be used in the QM/MM calculations. The same molecular mechanisms were obtained although slightly higher energy barriers were observed for the rate-limiting steps, that is, the first step (ca. 1.5–4.5 kcal mol^{-1}). We also obtained the PMF values in aqueous solution using the same QM/MM simulation protocol than for the antibody-catalyzed reaction. In this case, the calculated free energy barrier was 2.5 kcal mol^{-1} higher than the experimental value.[8] We think that this is a reasonable estimation of the expected error in the catalytic free energy barriers.
[21] Once the full PMF values were obtained for the oxy-Cope rearrangement (Figure 1) MD trajectories of 1 ns were carried out in the region of the MC and TS (with constraints applied just in the reaction coordinate of the latter), as well as for the TSA–CA complexes. The values mentioned in the text come from the averaged distances obtained in these long QM/MM trajectories.
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