Article

Enzymatic Effects on Reactant and Transition States. The Case of Chalcone Isomerase

J. Javier Ruiz-Perna, Estanislao Silla, and Iaki Tun

J. Am. Chem. Soc., **2007**, 129 (29), 9117-9124• DOI: 10.1021/ja071720+ • Publication Date (Web): 28 June 2007

J|A|C|S
ARTICLES
Published on Web 06/28/2007

Enzymatic Effects on Reactant and Transition States. The Case of Chalcone Isomerase

J. Javier Ruiz-Pernía, Estanislao Silla, and Iñaki Tuñón*

Contribution from the Departamento de Química Física, Universitat de València, 46100 Burjassot, Spain

Received March 12, 2007; E-mail: tunon@uv.es

Abstract: Chalcone isomerase catalyzes the transformation of chalcone to naringerin as a part of flavonoid biosynthetic pathways. The global reaction takes place through a conformational change of the substrate followed by chemical reaction, being thus an excellent example to analyze current theories about enzyme catalysis. We here present a detailed theoretical study of the enzymatic action on the conformational pre-equilibria and on the chemical steps for two different substrates of this enzyme. Free-energy profiles are obtained in terms of potentials of mean force using hybrid quantum mechanics/molecular mechanics potentials. The role of the enzyme becomes clear when compared to the counterpart equilibria and reactions in aqueous solution. The enzyme does not only favor the chemical reaction lowering the corresponding activation free energy but also displaces the conformational equilibria of the substrates toward the reactive form. These results, which can be rationalized in terms of the electrostatic interactions established in the active site between the substrate and the environment, agree with a more general picture of enzyme catalysis. According to this, an active site designed to accommodate the transition state of the reaction would also have consequences on the reactant state, stabilizing those forms which are geometrically and/or electronically closer to the transition structure.

1. Introduction

Enzymes increase the rate constant of chemical processes by several orders of magnitude.[1] Besides this fundamental observation, the origin of the enzymatic catalytic efficiency is still a quite controversial question.[2−5] Roughly speaking, two different theories, with several variants, have been elaborated to understand enzymatic activity. The first of them stress the effect of the enzyme on the reaction transition state (TS). According to these TS theories energy barrier reduction is attained by means of TS stabilization relative to the uncatalyzed process, the counterpart reaction in aqueous solution.[6−8] This stabilization would be mainly due to the electrostatic interactions established between the substrate and the catalytic active site, which would be preorganized to this end. Water molecules would be also capable of strong stabilization for those reactions in which the TS is more polar than the reactant state (RS) but a considerable energetic prize must be paid owing to the reorganization of the water structure around the solute. The second approach explains the rate constant enhancement reached by enzymes on the basis of a RS destabilization.[9−13] Thus, the enzymatic environment would push the reactant molecules toward the TS in an electronic or geometrical sense. According to this, enzymes would favor the formation of especially reactive conformations (near-attack conformations or NACs).[13] Recently, some of us proposed that RS destabilization could be related to TS stabilization, considering that an active site complementary to the TS would also have consequences on the RS, favoring that conformation electronically and/or geometrically closer to the TS.[14] In a similar way, recent works[3] show and discuss that the two concepts are physically equivalent within the limits of transition-state theory (TST) and amount only to two different descriptions of TS stabilization.

Chorismate mutase, which catalyzes the transformation of chorismate to prephenate, has been extensively used to analyze these theories on the enzymatic action.[15−27] This enzyme

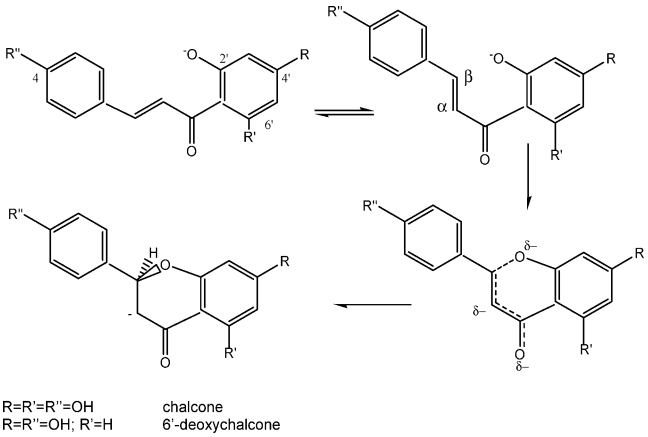
(1) Wolfenden, R.; Snider, M. J. *Acc. Chem. Res.* **2001**, *34*, 938−945.
(2) Benkovic, S. J.; Hammes-Schiffer, S. *Science* **2003**, *301*, 1196−1202.
(3) García-Viloca, M.; Gao, J.; Karplus, M.; Truhlar, D. G. *Science* **2004**, *303*, 186−195.
(4) Houk, K. N.; Leach, A. G.; Kim, S. P.; Zhang, X. Y. *Angew. Chem., Int. Ed.* **2003**, *42*, 4872−4897.
(5) Bruice, T. C.; Bruice, P. Y. *J. Am. Chem. Soc.* **2005**, *127*, 12478−12479.
(6) Villá, J.; Warshel, A. *J. Phys. Chem. B* **2001**, *105*, 7887−7907.
(7) Warshel, A. *J. Biol. Chem.* **1998**, *273*, 27035−27038.
(8) Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5250−5254.
(9) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678.
(10) Kollman, P. A.; Kuhn, B.; Donini, O.; Perakyla, M.; Stanton, R.; Bakowies, D. *Acc. Chem. Res.* **2001**, *34*, 72−79.
(11) Mesecar, A. D.; Stoddard, B. L.; Koshland, D. E. *Science* **1997**, *277*, 202−206.
(12) Menger, F. M. *Acc. Chem. Res.* **1993**, *26*, 206−212.
(13) Bruice, T. C. *Acc. Chem. Res.* **2002**, *35*, 139−148.
(14) Marti, S.; Roca, M.; Andres, J.; Moliner, V.; Silla, E.; Tuñón, I.; Bertrán, J. *Chem. Soc. Rev.* **2004**, *33*, 98−107.
(15) Martí, S.; Andrés, J.; Moliner, V.; Silla, E.; Tuñón, I.; Bertrán, J. *J. Am. Chem. Soc.* **2004**, *126*, 311−319.
(16) Lee, Y. S.; Worthington, S. E.; Krauss, M.; Brooks, B. R. *J. Phys. Chem. B* **2002**, *106*, 12059−12065.
(17) Martí, S.; Andrés, J.; Moliner, V.; Silla, E.; Tuñón, I.; Bertrán, J.; Field, M. J. *J. Am. Chem. Soc.* **2001**, *123*, 1709−1712.
(18) Kast, P.; Tewari, Y. B.; Wiest, O.; Hilvert, D.; Houk, K. N.; Goldberg, R. N. *J. Phys. Chem. B* **1997**, *101*, 10976−10982.
(19) Crespo, A.; Scherlis, D. A.; Marti, M. A.; Ordejon, P.; Roitberg, A. E.; Estrin, D. A. *J. Phys. Chem. B* **2003**, *107*, 13728−13736.
(20) Chook, Y. M.; Gray, J. V.; Ke, H. M.; Lipscomb, W. N. *J. Mol. Biol.* **1994**, *240*, 476−500.
(21) Acevedo, O.; Jorgensen, W. L. *Org. Lett.* **2004**, *6*, 2881−2884.
(22) Mulholland, A. J. *Drug Discovery Today* **2005**, *10*, 1393−1402.

10.1021/ja071720+ CCC: $37.00

presents some important advantages for the theoretical analysis. In particular, the reaction mechanism is intramolecular in aqueous solution and in the enzyme and then does not involve any residue or water molecule during the chemical reorganization. Second, the role of the environment on the RS can be discussed in terms of a preequilibrium between pseudo-diequatorial and pseudo-diaxial forms of chorismate, the former being geometrically closer to the TS.[28] Discussing in terms of different reactant conformations, with different chemical properties, avoids the use of the NAC concept, which always contains a certain degree of arbitrariness in its definition. Obviously, when the substrate comes into the active site in its RS the active site can also induce displacements in the conformational equilibrium between diequatorial and diaxial forms of chorismate. The stabilization of a particular state (such as the TS) obviously affects the stability of other neighboring states (reactant conformational states). Anyway, it seems clear that the maximum activity of the enzyme, in terms of interaction energy with the substrate, is played in the TS.[26−28] A nice recent analysis based on a kinetic model showed that chorismate mutase gains more catalytic efficiency by adaptation of its structure to TS stabilization rather than to RS destabilization.[29] As stated by the authors this result cannot be still considered as a general conclusion and needs to be verified in more examples.

Chalcone isomerase (CHI) offers an excellent example to gain a deeper insight in a general theory of enzymatic activity in terms of TS stabilization and RS destabilization. CHI plays a central role in flavonoid biosynthetic pathways, catalyzing the transformation of chalcone (4,2′,4′,6′-tetrahydroxychalcone) and 6′-deoxychalcone (4,2′,4′-trihydroxychalcone) into (2*S*)-naringerin (5,7,4′-trihydroxyflavanone) and (2*S*)-5-deoxyflavanone (7,4′-dihydroxyflavanone),[30,31] Both substrates are partially deprotonated at the 2′ position in physiological conditions in aqueous solution,[31] a requisite to produce an intramolecular Michael addition to the α,*β*-double bound[31] (see Scheme 1). This is probably the rate-limiting step of the process, which is followed by protonation of the resulting carbanion. Both chalcone and 6′-deoxychalcone spontaneously suffer cyclization in solution giving enantiomeric mixtures, while CHI ensures the rapid formation of the biological active (2*S*)-flavanones operating near the diffusion-controlled limit.[31] Interestingly, the 2′-oxyanion can exist in at least two different conformations, the productive *s*-trans conformer and the unproductive *s*-cis conformer. Conformational investigations of related α,*β*-unsaturated ketones in solution show that these compounds exist in both conformations but that the equilibrium lies toward the *s*-cis conformer.[32] Thus, a general perspective on this chemical

Scheme 1



transformation should also consider the chemical preequilibrium between reactant conformers.

In a recent study Bruice and co-workers carried out an analysis of the conformational equilibrium between *s*-cis and *s*-trans conformers of the oxoanion of 6′-deoxychalcone in aqueous solution and in CHI active site.[33,34] These authors found that the free-energy difference between the most stable non-productive *s*-cis conformer and the productive *s*-trans conformer increased when passing from aqueous solution to the active site (from approximately 3 kcal/mol in solution up to 6 kcal/mol in CHI). This finding is obviously opposed to the conclusions reached in the case of chorismate mutase and the integrated view of TS stabilization and RS destabilization effects. There was, however, a limitation in this study because the 6′-deoxychalcone (and the environment) was described using a purely classical molecular mechanics (MM) potential, which could be too limited to describe highly delocalized and polarizable systems as the present one.

In a first approach to this enzyme we recently used current hybrid quantum mechanical/molecular mechanical (QM/MM) potentials to investigate the problem of CHI active-site geometry, as far as two possible conformations of the active site were previously described in the literature.[35] These conformations essentially differ in the positioning of residues Thr48 and Lys97 relative to the substrate. According to the potentials of mean force (PMFs) traced for the reaction step using both active-site conformations, the one presenting a smaller substrate−Lys97 distance also had a significantly smaller reaction free-energy barrier. This active-site conformation was selected as the most appropriate to describe the reaction in this work. To understand and rationalize the origin of the enzymatic efficiency we have now analyzed both the conformational preequilibrium between reactant conformations and the chemical transformation, comparing these to the counterpart processes in aqueous solution. To verify the tendencies obtained from our simulations both chalcone and 6′-deoxychalcone have been considered as substrates. This enzyme offers a unique opportunity to discuss enzymatic effects in terms of reactant and transition-state effects, separating them unambiguously. According to our analysis, the role of CHI can be understood according to a general theory of

(23) Ranaghan, K. E.; Ridder, L.; Szefczyk, B.; Sokalski, W. A.; Hermann, J. C.; Mulholland, A. J. *Org. Biomol. Chem.* **2004**, *2*, 968−980.
(24) Warshel, A. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 425−443.
(25) Woodcock, H. L.; Hodoscek, M.; Sherwood, P.; Lee, Y. S.; Schaefer, H. F.; Brooks, B. R. *Theor. Chem. Acc.* **2003**, *109*, 140−148.
(26) Strajbl, M.; Shurki, A.; Kato, M.; Warshel, A. *J. Am. Chem. Soc.* **2003**, *125*, 10228−10237.
(27) Szefczyk, B.; Mulholland, A. J.; Ranaghan, K. E.; Sokalski, W. A. *J. Am. Chem. Soc.* **2004**, *126*, 16148−16159.
(28) Martí, S.; Andrés, J.; Moliner, V.; Silla, E.; Tuñón, I.; Bertrán, J. *Chem.−Eur. J.* **2003**, *9*, 984−991.
(29) Giraldo, J.; Roche, D.; Rovira, X.; Serra, J. *FEBS Lett.* **2006**, *580*, 2170−2177.
(30) Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol.* **2000**, *7*, 786−791.
(31) Jez, J. M.; Noel, J. P. *J. Biol. Chem.* **2002**, *277*, 1361−1369.
(32) Furlong, J. J. P.; Nudelman, N. S. *J. Chem. Soc., Perkin Trans. 2* **1985**, 633−639.
(33) Hur, S.; Bruice, T. C. *J. Am. Chem. Soc.* **2003**, *125*, 1472−1473.
(34) Hur, S.; Newby, Z. E. R.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 2730−2735.
(35) Ruiz-Pernía, J. J.; Silla, E.; Tuñón, I. *J. Phys. Chem. B* **2006**, *110*, 20686−20692.

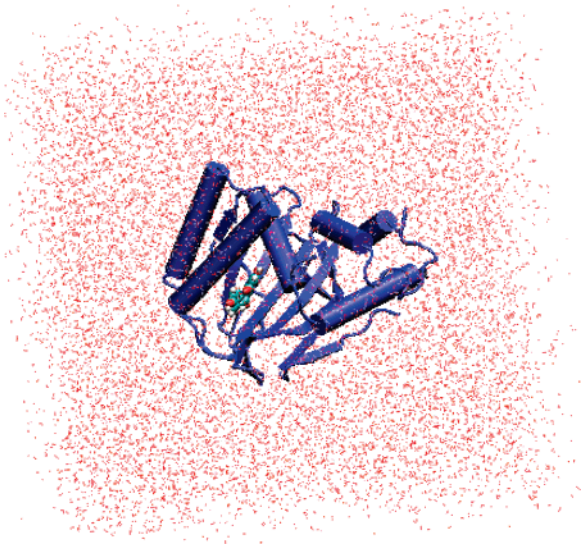


Figure 1. Snapshot of the system employed in the simulations with the enzyme place in the center of a simulation box containing water molecules. A chalcone molecule (balls and sticks) is placed in the active site.

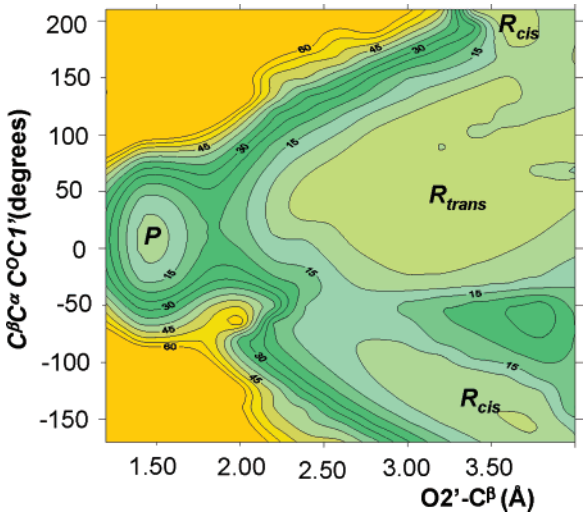


Figure 2. AM1/MM PES obtained for chalcone substrate in CHI active site. Isopotential energy lines are shown each at 5 kcal/mol. The position of the reactant in its *s*-cis (R_{cis}) and *s*-trans (R_{trans}) forms and of the reaction product (P) are approximately shown on the PES.

enzymes: catalysis is reached through an active site designed to fit to the transition state, which, in turn, also favors the conformational state of the reactants that is geometrically and/or electronically closer. The RS destabilization can be then rationalized in terms of TS stabilization.

2. Methodology

The initial coordinates of the system were taken from the X-ray crystal structure 1EYQ of CHI, with (2*S*)-naringenin in the active site which it is the product of the reaction from chalcone reactant.[30] In our previous work,[35] we analyzed two different active-site conformations, concluding that the best active is that presenting a direct interaction between Lys97 and the substrate. Here we used as a starting point the equilibrated system obtained in that work with the most reactive active-site geometry. We employed a hybrid quantum mechanics/molecular mechanics (QM/MM) description for our system. The reactant molecules constitute the QM subsystems, 31 atoms for chalcone and 30 for 6′-deoxychalcone, that were described using the AM1 Hamiltonian.[36] Hydrogen atoms were added to all the system using DYNAMO facilities.[37] Afterward, the system was placed inside a cubic box (79.5 Å of side) of water molecules centered on the QM subsystem. The MM subsystem was then formed by 3231 enzyme atoms, 591 crystallization water atoms, and 45972 solvation water atoms, described using the OPLS-AA[38,39] and TIP3P potentials.[40] A view of the simulation system is presented in Figure 1. During the QM/MM simulations we employed NVT and periodic boundary conditions and a cutoff radius of 13.5 Å for all kinds of interactions. To reduce computational cost, after equilibration all atoms beyond 24 Å of O2′ atom of the substrate were kept frozen. To compare the catalytic power of the CHI enzyme, we also studied the counterpart reaction in water. The system in water was formed by the substrate, the QM part, and a cubic box (55.5 Å of side) of TIP3P water molecules centered on the O2′ atom of the QM subsystem.

To know if the conformational equilibrium and the chemical transformation can be considered as independent processes we first traced 2-D potential energy surface (PES) for chalcone and 6′-deoxychalcone in the CHI active site using as distinguished coordinates the O2′−C$^{\beta}$ distance, which controls the Michael addition, and the C$^{\beta}$−C$^{\alpha}$−C^{O}−C1′ dihedral angle, which controls the *s*-cis/*s*-trans transformation (note that we changed the notation of the atoms with respect to our previous work[35] to accommodate to the more usual convention). Figure 2 shows the AM1/MM PES obtained for chalcone (the result for 6′-deoxychalcone being very similar).[41] This PES displays three different minima corresponding to the chalcone in its *s*-cis and *s*-trans forms and to naringerin (the product of the Michael addition). According to this PES it can be concluded that the reaction step occurs through the *s*-trans conformer, while both conformational forms of chalcone (and of 6′-deoxychalcone) can be described as energy minima in the CHI active site.

To analyze the energetics of the process we obtained the corresponding potentials of mean force (PMFs)[42] by means of a series of molecular dynamics (MD) simulations in which the reaction coordinate was constrained. For the reaction step, the O2′−C$^{\beta}$ distance was the natural choice. This variable represented very closely the minimum energy paths that were found in the enzyme and in the gas phase. The different values of the variable sampled during the simulations were then pieced together by means of the weighted histogram analysis method (WHAM)[43] to construct the full distribution function from which the PMF was obtained. The values of the force constant used for the harmonic umbrella sampling (2500 kJ mol^{-1} Å^{-2} on the reaction coordinate for the enzyme system and 3500 kJ mol^{-1} Å^{-2} for the water system) were determined to allow a full overlapping of the different windows traced in the PMF evaluation, but without losing control over the selected coordinate. The windows were run in a consecutive way starting from a transition structure toward reactants and products. Each window was started from the final configuration of the precedent window and consisted of 2 ps of equilibration followed by 10 ps of

(36) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902−3909.
(37) Field, M. J.; Albe, M.; Bret, C.; Proust-De Martin, F.; Thomas, A. *J. Comput. Chem.* **2000**, *21*, 1088−1100.
(38) Jorgensen, W. L.; Tirado-Rives, J. *J. Am. Chem. Soc.* **1988**, *110*, 1657−1666.
(39) Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. L. *J. Phys. Chem. B* **2001**, *105*, 6474−6487.
(40) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. *J. Chem. Phys.* **1983**, *79*, 926−935.
(41) A potential energy surface was calculated for each system (both substrates in enzyme and water systems). The O2′−C$^{\beta}$ distance and the C$^{\beta}$−C$^{\alpha}$−C^{O}−C1′ dihedral angle were chosen as the variables defining these PESs. The distance was varied from 1.4 to 3.0 in steps of 0.1 Å, and the dihedral angle was changed from 0 to 360° in steps of 20°, so a total of 17 × 18 points were calculated. For each of these points, both coordinates were kept frozen while the rest of the system was minimized, following GRACEFULL algorithm (Martí, S.; Moliner, V.; Tuñón, I. *J. Chem. Theor. Comput.* **2006**, *2*, 216−216.) implemented in DYNAMO.
(42) Roux, B. *Comput. Phys. Commun.* **1995**, *91*, 275−282.
(43) Torrie, G. M.; Valleau, J. P. *J. Comput. Phys.* **1977**, *23*, 187−199.

production using a reference temperature of 300 K. The total number of windows employed to cover the whole range of the reaction coordinate from reactants to products was 61, both in the enzyme and in aqueous solution. Thus, a total of four PMFs are obtained, two for chalcone (in water and enzyme systems) and two for 6′-deoxychalcone (in water and enzyme systems).

As we described in our previous paper,[35] the AM1 description of the QM part leads to a noticeable overestimation of the reaction free-energy barriers. Then, we decided to obtain the PMFs associated to the Michael addition of the nucleophilic oxygen over the α,β-double bond using the interpolated corrections (IC) method.[44,45] This is an extension of the interpolated corrections methodology developed by Truhlar et al.[46−48] for gas-phase dynamical calculations. In these methods an energy correction term is added to the total potential energy:

$$E_T = E_{QM} + E_{QM/MM} + E_{MM}$$

where the terms appearing on the right-hand side of the equation represent the energy of the QM subsystem, the interaction energy between the two subsystems (including the polarization of the QM subsystem), and the self-energy of the MM part, respectively. The correction energy term is written as a function of the distinguished reaction coordinate employed to construct the PMF, in this case the O2′−C$^{\alpha}$ distance. The correction term is evaluated as a single-point energy difference between a low-level method and a high-level one (in this case AM1/MM and MP2/6-31+G(d,p)/MM, respectively). The structures chosen for this single-point energy difference (a total of 21, including the transition structure, reactants, and products) were taken from the IRC traced at the AM1/MM level (one IRC was traced for each substrate in each of the two media). Finally, to have a continuous and derivable energy function the correction energy term is interpolated through the use of cubic splines.[49] Thus, the final corrected potential-energy function used in the evaluation of the PMF associated to the Michael addition is

$$E_T^{corr} = E_T^{AM1}(r_{QM},r_{MM}) + \text{spline}[(\Delta E(d_{O2'-C\beta}))]$$

where $\Delta E = E_T^{MP2/MM} - E_T^{AM1/MM}$ and r_{QM} and r_{MM} indicate the position of the QM atoms and MM centers.

To analyze the conformational equilibrium between unproductive (*s*-cis) and productive (*s*-trans) forms of the substrate in the CHI active site and in solution we obtained the PMFs associated to the C$^{\beta}$−C$^{\alpha}$−C^{O}−C1′ dihedral angle. The different values of the variable sampled during MD simulations were pieced together by WHAM to construct the full distribution function from which the PMF was obtained. The value of the force constant used for the harmonic umbrella sampling was 0.5 kJ mol^{-1} deg^{-2} for the enzymatic and aqueous solution systems. The windows were run in a consecutive way starting from the reactive form of the substrate (*s*-trans conformer) obtained from the PMF traced using the O2′−C$^{\beta}$ distance. Each window was started from the final configuration of the precedent window and consisted of 2 ps of equilibration followed by 30 ps of production, because hysteresis was detected when shorter simulation times were used. This was long enough to sample a wide range of structures at a reference temperature of 300 K. The total number of windows employed to cover the whole range of the coordinate from the *s*-trans conformer to the *s*-cis one was 63 (the reference value of the dihedral angle was changed in 3 deg at each successive simulation window). A total of four PMFs were obtained, two for chalcone (in water and in the enzyme) and two for 6′-deoxychalcone using a reference temperature of 300 K.

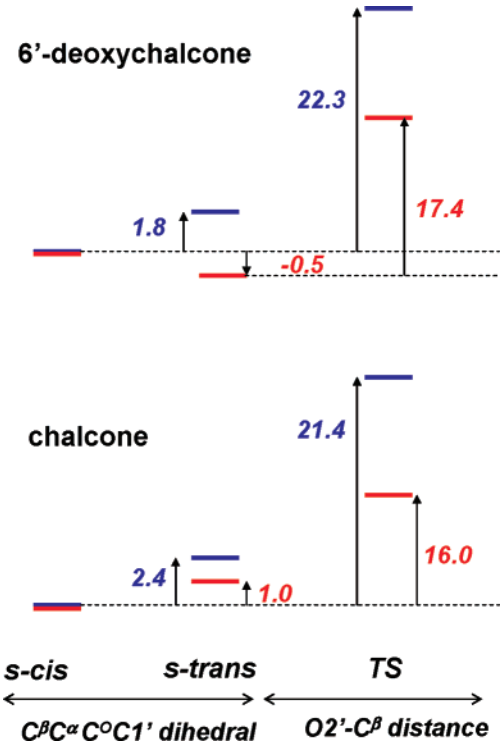


Figure 3. Free-energy profiles (obtained as PMF differences) in kcal/mol for the conformational equilibrium and chemical reaction of 6′-deoxychalcone and chalcone in water solution (blue) and in CHI (red).

Table 1. Calculated and Experimentally-Derived Free-Energy Barriers (in Kcal/mol) at 25 °C for the Michael Addition of the Deprotonated Forms of Chalcone and 6′-Deoxychalcone in Aqueous Solution and in CHI

	chalcone		6′-deoxychalcone	
	water	CHI	water	CHI
$\Delta G^{\ddagger}_{calcd}$	21.4	16.0	22.3	17.4
$\Delta G^{\ddagger}_{exp}$	23.1[a]	14.4[a]	25.1[a]; 21.7[b]	16.0[a]

[a] Reference 31. [b] Reference 50.

3. Results

Free-Energy Profiles. Figure 3 schematically displays the relative free energies of the relevant states obtained in terms of PMFs associated to the dihedral driven transformation between the *s*-cis and *s*-trans conformers of chalcone and 6′-deoxychalcone both in aqueous solution and in the CHI active site together with the transformation from the *s*-trans form to the transition state of the Michael addition. Combining these two processes, the global free-energy barriers obtained as PMF differences for the reaction of the deprotonated forms of chalcone and 6′-deoxychalcone in aqueous solution and in CHI are given in Table 1 together with experimental data.[31,50] Experimental free-energy barriers were obtained from application of the transition state theory formula to the first-order rate constants. We think that the difference observed between the two experimental values corresponding to the reaction of 6′-deoxychalcone in aqueous solution could be due to the fact that different pH conditions were used in the kinetic measurments.[31,50] The free-

(44) Ruiz-Pernía, J. J.; Silla, E.; Tuñón, I.; Martí, S.; Moliner, V. *J. Phys. Chem. B* **2004**, *108*, 8427−8433.
(45) Ruiz-Pernía, J. J.; Silla, E.; Tuñón, I.; Martí, S. *J. Phys. Chem. B* **2006**, *110*, 17663−17670.
(46) Corchado, J. C.; Coitino, E. L.; Chuang, Y. Y.; Fast, P. L.; Truhlar, D. G. *J. Phys. Chem. A* **1998**, *102*, 2424−2438.
(47) Chuang, Y. Y.; Corchado, J. C.; Truhlar, D. G. *J. Phys. Chem. A* **1999**, *103*, 1140−1149.
(48) Nguyen, K. A.; Rossi, I.; Truhlar, D. G. *J. Chem. Phys.* **1995**, *103*, 5522−5530.
(49) Renka, R. J. *ACM Trans. Math. Software* **1993**, *19*, 81−94.
(50) Furlong, J. J. P.; Nudelman, N. S. *J. Chem. Soc., Perkin Trans. 2* **1988**, 1213−1217.

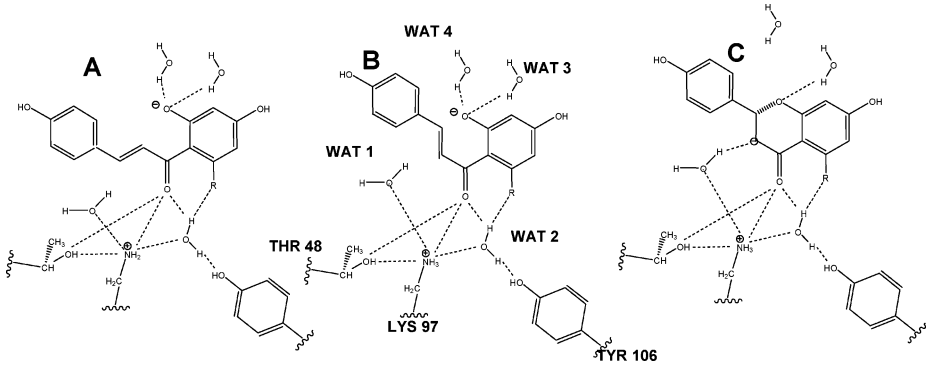


Figure 4. Schematic view of the most important substrate-enzyme interactions established in the reactant state (*s*-cis conformer (A) and *s*-trans conformer (B)) and transition state (C).

energy barrier derived from the rate constant obtained at pH = 7.5 is 25.1 kcal/mol,[31] while using a rate constant measured at larger pH values (in the pH-independent region) we estimated a free-energy barrier of 21.7 kcal/mol.[50] We attribute this discrepancy to the fact that in the former case probably not all the substrate was ionized, which translates into a larger phenomenological free-energy barrier. So, our theoretical value, obtained for a completely ionized substrate, compares better with the latter experimental estimation. This observation could also explain the difference between the experimental and the theoretical activation free-energy values obtained for chalcone in solution, as far as the experimental value was also determined at pH = 7.5.

The PMFs associated to the change in the dihedral angle in solution show that the *s*-cis conformations are more stable in both cases (chalcone and 6′-deoxychalcone) in agreement with previous interpretation of experimental and theoretical data.[32] The absolute minima appear at dihedral angles of about 130° and 150° for 6′-deoxychalcone and chalcone, respectively, while the *s*-trans minima presents a dihedral angle of about 50° in both cases. The free-energy barrier in between both minima measured from the highest energy conformer (that is, for the *s*-trans → *s*-cis transformation) are of only 0.3 kcal/mol; thus, the *s*-trans conformers in aqueous solution should be considered as very shallow minima, at least at the AM1/TIP3P level of theory. In the work of Hur et al.[34] on 6′-deoxychalcone a very similar free-energy difference was obtained, but the computed free-energy barrier for the *s*-trans → *s*-cis change was of about 6 kcal/mol. We attribute this large difference to the fact that a simpler pure MM potential was used in that work and also to the fact that the dihedral parameters were derived from a system were phenyl rings were substituted by methyl groups and then delocalization effects were not properly accounted for.

According to our results, the effect of CHI on the preequilibrium is to favor, relative to the solvent, the *s*-trans conformer. The free-energy differences are reduced from 2.4 to only 1.0 kcal/mol in the case of chalcone, while for 6′-deoxychalcone the *s*-trans conformer becomes the most stable one in the CHI active site. The *s*-trans minima appear at dihedral angle values of 10° and 30° for 6′-deoxychalcone and chalcone, respectively, while the *s*-cis minima appear at 120° and 130°. The free-energy barriers for the *s*-trans→ *s*-cis transformation derived from our PMFs are of only 0.5 and 1.5 kcal/mol in the case of chalcone and 6′-deoxychalcone, respectively. It must be noted that these results agree with the hypothesis of a preorganization of the substrate favoring those conformations closer to the transition state of the reaction to be catalyzed, in this case the *s*-trans conformer, relative to the same process in aqueous solution.[14,26,29] The results of Hur et al. show a completely different trend.[34] According to their results the free-energy difference between the *s*-cis and *s*-trans conformers is increased up to 6 kcal/mol in CHI active site, while the computed free-energy barrier measured from the *s*-trans conformer was of about 4 kcal/mol.

With respect to the chemical reaction, the intramolecular nucleophilic attack of the oxyanion to the double bond, the process presents a significantly lower free-energy barrier in CHI than in aqueous solution. The computed free-energy barriers presented in Table 1 correspond to the reaction starting from the most stable conformer of the deprotonated substrate. As stated before, when comparing to experimental data obtained in solution it should be taken into account that, depending on the experimental pH conditions,[30−32,50,51] a significant contribution due to the pK_a of the substrate could be observed. Anyway, our computed free-energy barriers (directly derived from the PMFs) compare reasonably well with experimental data and do not only reproduce the catalytic effect of the enzyme but also the relative ordering between 6′-deoxychalcone and chalcone.

Analysis of the Conformational Equilibrium. We will try to highlight into the origin of enzymatic effects by inspection of the specific interactions established in the active site between the substrate (in its different states) and the protein. Figure 4 schematically shows the more important contacts observed in the reactant state (both for the *s*-cis and the *s*-trans conformer) and in the transition state.

In our preceding work[35] we investigated on the existence of different active-site geometries observed in X-ray diffraction data.[30,31,52] The two possibilities essentially differ in the disposition of Thr48 and Lys97. In the active site displaying a larger catalytic power, the one selected for this study, Lys97 is close to the substrate, establishing an interaction through the carbonyl oxygen of the substrate and a hydrogen-bond interaction with Thr48. In the other one an additional water molecule appears in the active site and the Lys97−substrate interaction is no longer direct but water-mediated.[35] Other important residues are Tyr106, that interacts with the substrate through a water molecule (either with the carbonyl group or the 6′-hydroxy group in the case of chalcone), Thr190 (not shown in the figure) that forms a hydrogen bond with the 4′-hydroxyl group of the

(51) Miles, C. O.; Main, L. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1639−1642.
(52) Jez, J. M.; Bowman, M. E.; Noel, J. P. *Biochemistry* **2002**, *41*, 5168−5176.

substrate and Asn113 (not shown) that may also form a hydrogen bond with the 2′-oxyanion. In addition, up to four water molecules are found in the active site, three of them are crystallographic molecules and another one (labeled as Wat1) comes form the solvation shell. This last water molecule plays an important role in the subsequent proton transfer to the carbanion to produce the final reaction product. As discussed below and elsewhere[34,35] one of the water molecules (Wat4) is released from the active site during the chemical reaction.

To understand the enzymatic effect on the *s*-cis/*s*-trans conformational equilibria, we have focused our analysis on the substrate−enzyme interactions established through the O2′ oxyanion. Changes in the 2-hydroxy group are of minor importance as this phenyl group is always exposed to the solvent and thus we did not find specific interactions through this group while the evolution of the interactions established by the oxyanion are quite different in both media. In aqueous solution, the conformational change from *s*-cis to *s*-trans is accompanied by a partial desolvation of the O2′ oxyanion because this atom is less solvent-exposed in the *s*-trans conformer. The water oxygen-O2′ radial distribution functions obtained both for 6′-deoxychalcone and chalcone show a diminution in the number of water molecules in the first solvation shell. Integration of the first peak (with a maximum at 2.8 Å) leads to 4.5 water molecules in the first solvation shell of the *s*-cis conformer and 3.3 in the *s*-trans one, in both substrates. This desolvation obviously means an energy penalty contributing to the global energetic of the *s*-trans/*s*-cis equilibria. In the active site of CHI the situation is quite different. Rotation around the $C^{\alpha}C^{O}$ bond leads to a better placement of the substrate in the active site and some key interactions are reinforced. In the case of the O2′ oxyanion we have found three different hydrogen bonds in the active site, with two crystallographic water molecules (Wat3 and Wat4) and with the NH_2 moiety of Asn113. All these hydrogen bonds are shorter in the *s*-trans conformer than in the *s*-cis one. For 6′-deoxychlacone Wat3/Wat4/Asn113 averaged distances to O2′ oxyanion are reduced from 3.3/3.3/6.1 Å in the *s*-cis conformer to 2.9/2.8/3.4 Å in the *s*-trans form. In the case of chalcone, distances are reduced from 5.0/5.3/4.7 Å to 2.8/2.8/3.5 Å. We must keep in mind that when comparing to the substrate in aqueous solution, the O2′ oxyanion has been partially desolvated, loosing some hydrogen-bond interactions (from four to five hydrogen-bond contacts for the *s*-cis conformer in solution to only three in the enzyme). This obviously must be compensated with other interactions to have a favorable binding process. The binding free energies derived from the experimental K_m values are −5.4 and −6.9 kcal/mol for chalcone and 6′-deoxychalcone, respectively.[31] Thus, according to our free-energy calculations presented in Figure 3 both reactant conformers, the productive and the nonproductive forms, are stabilized in the enzymatic environment. One of the more important stabilizing interactions is that established between the carbonyl group and the positively charged Lys97. Considering the environmental effect on the *s*-trans/*s*-cis equilibria, the enzyme favors the productive *s*-trans form relatively to the aqueous solution equilibria, because the oxyanion is less desolvated in the *s*-trans form than in the *s*-cis form. Wat2 also contributes to the stabilization of the *s*-trans form relative to the *s*-cis conformer. This water molecule establishes a hydrogen bond with the carbonyl oxygen atom of 6′-deoxychalcone that is strengthened during the conformational change, being the donor−acceptor distance reduced from an averaged distance of 4.1 Å in the *s*-cis conformer to 2.7 Å in the *s*-trans one. In the case of chalcone, this water molecule interacts with the substrate through the 6′-hydroxyl group, and the averaged oxygen−oxygen distance is reduced from 3.6 to 3.4 Å when passing from the *s*-cis to the *s*-trans conformer.

Table 2. Averaged Mulliken Charges (in au) on Selected Atoms of the Substrate in the Reactant and Transition States in Aqueous Solution and in the Enzyme[a]

	chalcone				6′-deoxychalcone			
	RS_w	TS_w	RS_{enz}	TS_{enz}	RS_w	TS_w	RS_{enz}	TS_{enz}
O2′	−0.61	−0.54	−0.57	−0.45	−0.54	−0.38	−0.61	−0.52
C^{β}	0.20	0.33	−0.10	0.26	0.04	0.40	−0.08	0.23
C^{α}	−0.10	−0.33	−0.18	−0.60	0.00	−0.49	−0.21	−0.57
O	−0.43	−0.51	−0.51	−0.58	−0.44	−0.61	−0.53	−0.57

[a] Note that hydrogen atoms charge has been added to their neighbor atoms.

Analysis of the Chemical Reaction. From the electronic point of view, the Michael addition of the O2′ oxyanion to the α,*β*-double bond has been usually described as an electron transfer from the oxyanion to the carbonyl oxygen atom to form the corresponding enolate. However, we have recently shown, through analysis of averaged partial charges, that the reaction is better described as an electron flow toward the C^{α} atom to form the corresponding carbanion (note again the change in the notation of the atoms with respect to our previous work).[35] Averaged Mulliken charges obtained for the (*s*-trans) reactants state and for the transition state in aqueous solution and in CHI are provided in Table 2. We can observe that in all cases, the charge on the C^{α} in the TS increases (in absolute values) substantially more than on the carbonyl oxygen atom (O). According to this picture, interactions established through the carbonyl group are not expected to play a fundamental role in lowering the free-energy barrier as they should affect similarly to the reactants and the transition state. Otherwise, interactions with the C^{α} atom can play a decisive role increasing the catalytic rate constant. In this sense, positioning of Wat1 close to this carbon atom (see Figure 4) should be essential to assist the electron transfer and to act as proton donor in the subsequent step to give the final reaction product.

To investigate the differences in the interactions established between the substrate and the environment in aqueous solution and in the enzyme and to relate these differences to the catalytic power of the enzyme, we have computed the averaged electrostatic potential created by the MM atoms on selected atomic positions of the substrate. This provides a simple way to analyze the electrostatic effect of the environment on the reaction.[53,54] A positive value means that the environment favors the localization of negative charge on that particular atomic position. The averaged values of the electrostatic potential on selected atoms of the reactant state (*s*-trans) and the transition state in solution and in CHI are provided in Table 3.

In both media the electrostatic potential is, in all cases, positive, as expected from an environment adapted to accommodate a negatively charged substrate. In CHI we have a

(53) Roca, M.; Martí, S.; Andrés, J.; Moliner, V.; Tuñón, M.; Bertrán, J.; Williams, A. H. *J. Am. Chem. Soc.* **2003**, *125*, 7726−7737.
(54) Soriano, A.; Silla, E.; Tuñón, I.; Ruiz-Lopez, M. F. *J. Am. Chem. Soc.* **2005**, *127*, 1946−1957.

Table 3. Averaged Values of the Electrostatic Potential (in au) Created by the MM Environment on Selected Atomic Positions of the Substrate in the Reactant and Transition States in Aqueous Solution and in the Enzyme

	chalcone				6′-deoxychalcone			
	RS_w	TS_w	RS_{enz}	TS_{enz}	RS_w	TS_w	RS_{enz}	TS_{enz}
O2′	0.34	0.29	0.41	0.30	0.33	0.18	0.48	0.36
C^{β}	0.19	0.24	0.33	0.35	0.16	0.17	0.34	0.31
C^{α}	0.19	0.22	0.29	0.29	0.16	0.23	0.28	0.33
O	0.22	0.31	0.40	0.41	0.21	0.34	0.44	0.57

positively charged residue (Lys97) in the active site, while in solution this positive potential is a consequence of the reorientation of water molecules. It is interesting to note that the electrostatic potential on the O2′ oxyanion is more positive in the enzyme than in solution. This means that when comparing the reactive form of the reactant (*s*-trans conformer) in solution and in the active site we have found no evidence of desolvation of the nucleophilic group in the latter environment. The desolvation hypothesis has been postulated to explain catalysis in other reactions of the S_N2 type because a desolvated nucleophilic group is more reactive.[55] However, this hypothesis has been also strongly criticized.[56] In our case, for the *s*-trans conformer, we found a more positive potential on the oxyanion in the enzyme than in solution and this means stronger electrostatic interactions in the former media. The driving force of catalysis is not the desolvation of the O2′ oxyanion in the reactant state, at least if we compare the reaction barriers measured from the *s*-trans conformers. Of course, as discussed above, there is a loss of hydrogen-bond interactions if we compare the *s*-trans conformer in the enzyme to the conformational minima (*s*-cis) in solution but, according to our conformational PMFs the effect on the observed reaction free-energy barrier is moderate. In fact, according to our calculations on chalcone, the enzymatic effect on the reactant conformational equilibria would account for only for 1.4 kcal/mol (see Figure 3) of the total activation free-energy diminution (5.4 kcal/mol) observed when comparing the enzymatic and in solution barriers.

The question now is which is the origin for the rest of the catalytic effect? Or said in other words, why is the free-energy barrier, measured from the reactive (*s*-trans) conformer lower in CHI than in solution? When passing from the reactant state to the transition state the negative charge flows toward the C^{α} atom and the hydrogen-bond interactions of the O2′ atom are weakened and thus these interactions should contribute to increase the free-energy barrier. The averaged distances from the Wat3/Wat4 oxygen atoms to O2′ are increased from 2.8/2.8 Å in the reactant state of chalcone to 2.9/5.7 Å in the corresponding transition state. The hydrogen bond established with the N^H atom of Asn113 is also lengthened from 3.5 to 4.3 Å. In the case of 6′-deoxychalcone the hydrogen bond with Wat3 is slightly shortened (from 2.9 to 2.8 Å) while the one established with Wat4 is completely lost (the averaged distances passing from 2.8 Å in the reactant state to more than 6 Å in the transition state). The hydrogen bond with Asn113 is also weakened and the averaged distance increases from 3.1 Å in the reactant state to 3.4 Å in the transition state. The origin of the catalytic power of the enzyme is found, at least partly, in the interactions established through the C^{α} atom, which is developing a negative charge during the reaction process. Effectively the electrostatic potential created by the enzyme on this atom is more positive than in solution. Even in the enzymatic reactant state, a reaction stage in which the negative charge is still on the O2′ oxyanion, the electrostatic potential is already larger than the potential created by water molecules in solution in the corresponding transition state. This finding connects with the idea of a preorganized enzymatic environment prepared to accommodate the charge distribution of the transition state.[14,26] The main sources of this positive potential on the C^{α} atom in CHI are Wat1, a water molecule coming from the solvent shell, and the positively charged Lys97. In our previous work on CHI we concluded, comparing two different active-site conformations that, the proximity of Lys97 to C^{α} atom can account for up to 4 kcal/mol of the total barrier lowering caused by the enzyme,[35] being probably the largest source of the catalytic effect. From the reactant to the transition state the averaged distance from the substrate to Lys97 (measured from carbonyl oxygen to the N^H) diminishes by about 1 Å, increasing thus the stabilizing charge−charge interaction. The averaged distances from the oxygen atom of Wat1 to the C^{α} atom in the transition states of chalcone and 6′-deoxychalcone are 2.80 and 3.40 Å, respectively. The proximity of this water molecule is also essential for the last reaction step, in which the carbanion should be protonated to produce the final reaction product. We have not found a water molecule playing a similar role in aqueous solution, as revealed by the absence of a first solvation shell peak in the radial distribution function around the C^{α} atom in the transition state of the reaction. Anyway, a certain degree of reorientation of solvent molecules around this atom is observed when the transition state is reached, as reflected in the more positive value of the electrostatic potential created by water molecules on the C^{α} atom.

It is finally worth mentioning that the presence of a 6′-hydroxyl group in chalcone can account for the differences in the reactivity observed with respect to 6′-deoxychalcone. In aqueous solution, an intramolecular hydrogen bond with the carbonyl group stabilizes the flow of negative charge toward the C^{α} atom, as deduced from the comparison of the averaged charges obtained in the reactant states of chalcone and 6′-deoxychalcone in solution (see Table 2). In CHI this hydroxyl group is able to establish a hydrogen bond with Tyr106, and this interaction is noticeably stronger in the transition state than in the reactant state, thus making a favorable contribution to diminish the free-energy barrier.[35]

4. Conclusions

We have presented a QM/MM study of enzymatic effects on the conformational preequilibrium and reaction step of chalcone and 6′-deoxychalcone transformation into the corresponding flavanones, catalyzed by CHI. According to our results, chalcones may exist in at least two different conformational forms according to the relative disposition of the carbonyl group and the α,β-double bond. Only the *s*-trans conformer, which is not the most stable one in aqueous solution, is able to proceed up to the reaction products.

Both the conformational equilibria and the chemical reaction step have been analyzed tracing the PMFs associated to the C^{β}-C^{α}-C^{O}-C1′ dihedral angle and to the O2′- C^{β} distance, respec-

(55) Devi-Kesavan, L. S.; Gao, J. L. *J. Am. Chem. Soc.* **2003**, *125*, 1532−1540.
(56) Olsson, M. H. M.; Warshel, A. *J. Am. Chem. Soc.* **2004**, *126*, 15167−15179.

tively. Comparison with the PMFs obtained in aqueous solution has allowed us to clarify the role of the enzyme on both aspects of the global process. First, CHI favors the productive or reactive form of the reactants, this is the *s*-trans conformer, relative to what is found in aqueous solution. Second, the enzyme also significantly lowers the free-energy barrier for the Michael addition. Thus, the results agree with a more general picture of enzyme effects, according to which an active site designed to accommodate the transition state of the reaction to be catalyzed would also have consequences on the reactant state.[3,14,26,29] Reactions in which several conformations are available for the reactant molecules, such as chorismate rearrangement or the reaction analyzed here, are appropriate examples to analyze these effects as far as there is no need to introduce any arbitrary conformation along the reaction path such as NACs.[5] Enzyme effects, as compared to aqueous solution, can be discussed and analyzed in terms of well-defined states.

CHI catalyzes the Michael addition favoring the electron flow from the O2′ oxyanion to toward the C^{α} atom. This is accomplished by the presence of a positively charged Lys97 and a water molecule which is hydrogen bonded to the carbon atom in the transition state. It is also important to note that in this case desolvation of the oxyanion need not be invoked to explain catalysis. Desolvation of this atom is found if we compare the absolute energy minimum in aqueous solution (the *s*-cis conformer) with the reactive reactant form (*s*-trans) in the enzyme. However, the O2′ oxyanion in this *s*-trans conformer is effectively better solvated (as shown at least by the electrostatic potential) than in the same conformer in aqueous solution and the energy barrier measured from this conformer is substantially lower in the enzyme. Some hydrogen bonds between the environment and the O2′ atom are lost in the transition state but this is compensated by an approach of the substrate to Lys97 and the positioning of Wat1. In this way the enzyme is able to simultaneously lower the energy barrier and to displace the conformational equilibrium toward the productive form.

Acknowledgment. We are indebted to Ministerio Educación y Ciencia for project CTQ2006-15447-CO2-02 and Generalitat Valenciana for project GV06-021 and 865/2006, which supported this research. J.J.R.-P. thanks the Spanish Ministerio de Educación y Ciencia for a FPU doctoral fellowship.

JA071720+