Correlation of Calculated Activation Energies with Experimental Rate Constants for an Enzyme Catalyzed Aromatic Hydroxylation

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Received February 26, 1998
Revised Manuscript Received April 28, 1998

p-Hydroxybenzoate hydroxylase (PHBH, EC 1.14.13.2) is involved in the microbial degradation of aromatic compounds. It has become the model enzyme for the family of external flavoprotein monooxygenases.[1] In the present study, a combined quantum mechanical (QM) and molecular mechanical (MM) method is applied to calculate energy barriers for the hydroxylation of the parent substrate and four of its fluorinated analogues within the active site of PHBH. The energy barriers obtained show a clear correlation ($r = 0.96$) with experimentally determined rate constants for their enzymatic conversion, demonstrating the biochemical relevance of the present approach.

The aromatic hydroxylation catalyzed by PHBH has been proposed to proceed via the electrophilic attack of an intermediate hydroperoxide form of the flavin cofactor (C4a-hydroperoxyflavin) on the substrate C_3 (Figure 1).[2] This electrophilic attack is proposed to be the rate-limiting step at physiological conditions (25 °C, pH 8).[3] The crystal structure coordinates of PHBH in complex with the oxidized flavin cofactor and the substrate *p*-hydroxybenzoate[4] were used to build a three-dimensional model of the C4a-hydroperoxyflavin intermediate of PHBH. The hydroxylation reaction in this large system was studied by using a combined QM/MM potential function[5] which treated the flavin ring, modified to the reactive C4a-hydroperoxyflavin intermediate,[6] and the dianionic form of the substrate[2,3] quantum mechanically on the basis of the closed shell semiempirical AM1 Hamiltonian.[7] The steric and electronic influence of the surrounding enzyme was included by treating all other atoms molecular mechanically[8] using the CHARMm 22 force field,[9] describing polar hydrogens explicitly. For a correct description of the C1−C2 bond of the ribityl chain of the FAD cofactor, which crosses the QM/MM boundary, a "link atom"[5] was introduced. The coordinates of 330 water molecules in the crystal structure were used to build explicit water molecules using the HBUILD facility in CHARMM. Energy minimization was performed with the adopted basis Newton−Raphson method for all atoms within a 10 Å sphere around the distal oxygen of the cofactor, including the substrate, the C4a-hydroperoxyflavin, a number of amino acid residues, and 15 water molecules. All other atoms were fixed. A gradient tolerance of 0.01 kcal mol^{-1} Å^{-1} and a nonbonded cutoff of 11 Å were applied. The optimized model for the enzyme−C4a-hydroperoxyflavin−substrate complex intermediate was used as the starting geometry in a reaction pathway calculation for the attack of the C4a-hydroperoxyflavin on the substrate.

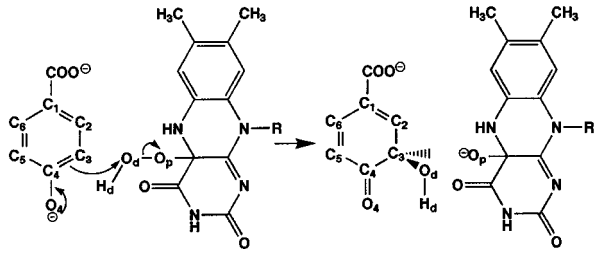

Figure 1. The electrophilic attack of the distal oxygen of the flavin cofactor on the substrate according to an electrophilic aromatic substitution mechanism. The cyclohexadienone formed converts into the final product 3,4-dihydroxybenzoate via keto−enol tautomerization.

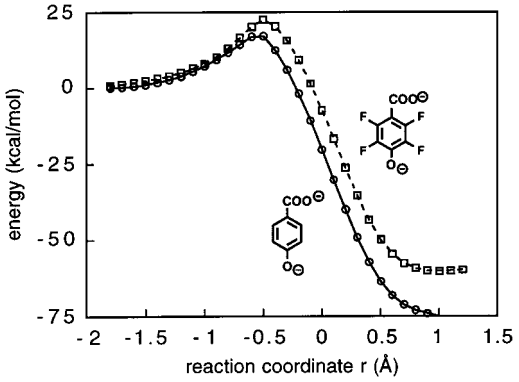

Figure 2. Energy profiles obtained for the electrophilic attack of the C4a-hydroperoxyflavin cofactor on *p*-hydroxybenzoate and 2,3,5,6-tetrafluoro-*p*-hydroxybenzoate. Energies are relative to the reactants.

A reaction coordinate (r), describing the breaking of the peroxide oxygen−oxygen bond (O_p-O_d) and the formation of the bond between the distal peroxide oxygen and the substrate C_3 (Figure 1), was applied by harmonically restraining ($k = 5000$ kcal mol^{-1} Å^{-2}) the difference in the lengths of these bonds. For each successive point along this reaction coordinate, energy minimization was performed as described above. The water molecules in the optimized region were harmonically restrained to their initial optimized positions ($k = 5.0$ kcal mol^{-1} Å^{-2}). Figure 2 presents the energies of the resulting pathway (solid line). The intermediate geometry at $r = -0.54$ is highest in energy and represents the approximate transition state structure of the hydroxylation step (Figure 3). Gas-phase normal-mode analysis[10] of this transition-state geometry resulted in one significant negative eigenvalue corresponding to the transfer of the hydroxy group from the cofactor to the substrate, which validates this transition-state geometry. The product geometry is a σ adduct of the *p*-hydroxybenzoate in which the C3 is changed from a planar (sp^2) to a tetrahedral (sp^3) conformation. Mulliken charges on the QM atoms in the reactant, transition state and product geometries are given in Table 1. The total charges on the substrate ring and cofactor reveal a net charge transfer of one electron from

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S0002-7863(98)00639-8 CCC: $15.00
Published on Web 07/15/1998

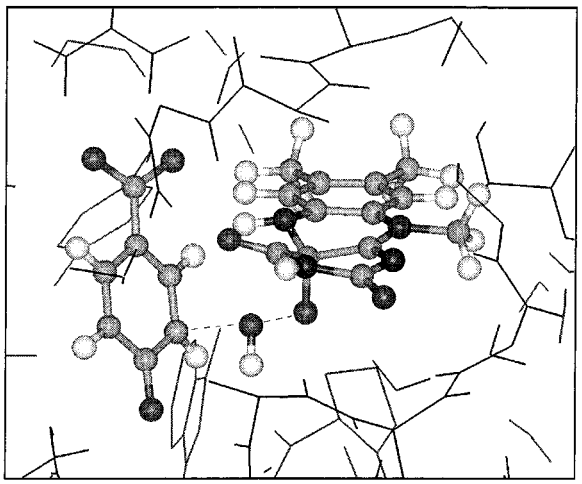

Figure 3. Approximate transition-state structure of the present reaction pathway model. Dotted lines indicate forming O_d-C_3 and breaking O_p-O_d bonds (Figure 1). Quantum mechanical atoms represented in ball-and-stick.

Table 1. Mulliken Atomic Charge Distribution in the Reactant, Transition-State, and Product Geometries[a]

atoms	reactants	transition state	products
flavin ring	0.10	0.00	−0.33
O_p	−0.19	−0.37	−0.66
O_d	−0.23	−0.13	−0.33
H_{O_d}	0.29	0.24	0.26
benzoate	−1.41	−1.21	−0.61
O_4	−0.56	−0.53	−0.32
total cofactor	−0.03		−1.00
total substrate	−1.97		−1.00

[a] Atom labeling is as in Figure 1.

the substrate to the C4a-hydroxyflavin (compare with Figure 1). Analysis of the geometrical changes in the substrate (results not shown) show that the C_1-C_2, C_5-C_6, and C_4-O_4 bond distances decrease whereas the other atomic distances in the carbon ring increase. Together with the observed decrease in charge on O_4, these results show the formation of a cyclohexadienone, indicating that the present model is in agreement with an electrophilic aromatic substitution type of mechanism as proposed on the basis of transient kinetic studies for a homologous flavin-dependent aromatic hydroxylase.[11]

The reaction pathways for four fluorinated substrate derivatives were calculated following the same procedure. In the initial structure, the substrate was placed such that the carbon atom to be hydroxylated is in the same position as C_3 of the native substrate. For all fluorinated substrates, each intermediate geometry obtained along the reaction coordinate was similar to the corresponding geometry of the reaction pathway with the native substrate, especially for the first part of the reaction coordinate toward the transition state (RMSD < 0.10 Å). However, the energy profiles obtained for the different substrates differ with respect to the height of the barrier (Figure 2). The total energy differences between the initial minimized geometry and the transition-state geometry vary from 17.6 to 22.5 kcal/mol for the various *p*-hydroxybenzoates, increasing with increasing number of fluorine substituents. These calculated energy barriers show a linear correlation ($r = 0.96$) with the natural logarithm of the experimental k_{cat} values for conversion of the different *p*-hydroxybenzoate derivatives by PHBH[12] (Figure 4). This correlation supports the proposal[3] that the electrophilic attack is rate limiting under physiological conditions.

Hydroxylation of some substrates by PHBH can be accompanied by a side reaction leading to hydrogen peroxide formation. It has been suggested that this uncoupling reaction could be related to the tendency of the flavin to adopt the so-called "out" conformation.[13] For the fluorinated substrates, however, this uncoupling reaction does not occur.[12,14]

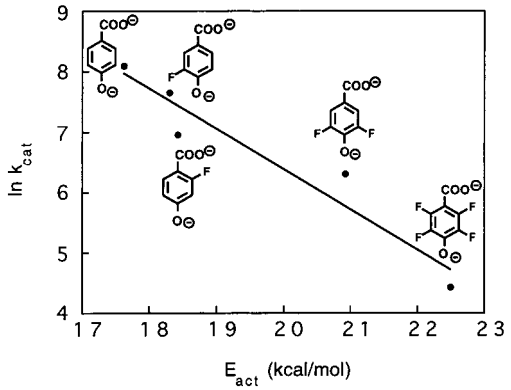

Figure 4. Linear correlation ($r = 0.96$) of experimental k_{cat} (min^{-1}) values for the overall hydroxylation of five *p*-hydroxybenzoate derivatives by PHBH (25 °C, pH 8)[12] with calculated energy barriers for the present reaction pathway model.

The activation energy for *p*-hydroxybenzoate has been experimentally determined to be around 12 kcal/mol.[15] This indicates that the calculated energy barriers are roughly a factor 1.5 too high in comparison to experimental data. Since the energy barriers are dominated by the energy difference in the quantum mechanical energy term (not shown), the absolute overestimation of the energy barriers probably lies in the semiempirical AM1 method which is known to overestimate absolute activation energies in many cases.[7] Relative activation energies, however, appear to be more reliable.[16] The QM/MM method has been applied in several enzyme studies.[17] To our knowledge the present study is the first demonstration of a correlation between calculated energy barriers and experimental rate constants for the enzymatic conversion of a series of substrates, which indicates that the QM/MM energy barriers are useful in a relative way. Furthermore, it indicates that studying a series of related fluorinated substrates provides a method to validate the QM/MM method. By using only fluorinated substrates, being similar in size compared to the nonsubstituted substrate, the present study was focused on the electronic effects of the substituents. Since steric effects are accounted for in the QM/MM method, the present approach may be extended to larger substituents. This indicates the potential of quantitative relationships between QM/MM energy barriers and experimental data to become a valuable tool in predicting rates of enzymatic conversions of new substrates.

Acknowledgment. This work was supported by The Netherlands Organization for Scientific Research (NWO). A.J.M. is a Wellcome Trust International Prize Travelling Research Fellow (grant no. 041229). We thank Prof. Martin Karplus for useful discussions.

Supporting Information Available: One table of calculated energy barriers (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA980639R

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