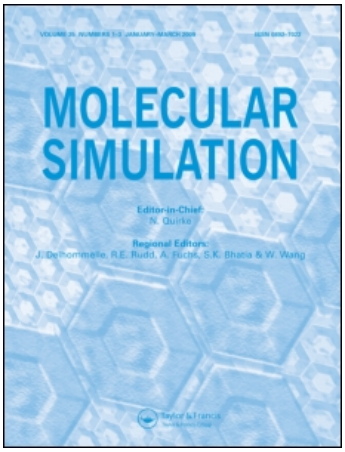

Molecular Simulation
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713644482

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J. C.-Basurto[ab]; J. Aburto[c]; J. T.-Ferrara[a]; E. Torres[d]
[a] Sección de Estudios de Posgrado e Investigación y Departamento de Bioquímica, Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico City, México [b] Departamento de Farmacología, Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico City, México [c] Instituto Mexicano del Petroleo, Mexico City, Mexico [d] Universidad Autónoma Metropolitana, Mexico City, México

To cite this Article C.-Basurto, J. , Aburto, J. , T.-Ferrara, J. and Torres, E.(2007) 'Ligand recognition by chloroperoxidase using molecular interaction fields and quantum chemistry calculations', Molecular Simulation, 33: 8, 649 — 654
To link to this Article: DOI: 10.1080/08927020701342041
URL: http://dx.doi.org/10.1080/08927020701342041

Molecular Simulation, Vol. 33, No. 8, July 2007, 649–654

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Ligand recognition by chloroperoxidase using molecular interaction fields and quantum chemistry calculations

J. C.-BASURTO†‡, J. ABURTO§, J. T.-FERRARA† and E. TORRES¶*

†Sección de Estudios de Posgrado e Investigación y Departamento de Bioquímica, Escuela Superior de Medicina, Instituto Politécnico Nacional, Plan de San Luis y Díaz Mirón, 11340 Mexico City, México
‡Departamento de Farmacología, Escuela Superior de Medicina, Instituto Politécnico Nacional, Plan de San Luis y Díaz Mirón, 11340 Mexico City, México
¶Universidad Autónoma Metropolitana, Unidad Cuajimalpa. Avenida Constituyentes 1054, 119950 Mexico City, México
§Instituto Mexicano del Petroleo, Eje Central Lazaro Cardenas 152, 07730 Mexico City, Mexico

(Received December 2006; in final form March 2007)

We recently reported that chloroperoxidase (CPO) from *Caldariomyces fumago* showed atypical kinetic behavior for the oxidation of 4,6 dimethyl dibenzothiophene (DMDBT). In this work, we undertake the theoretical study of DMDBT docking into CPO's active site, in order to clarify its binding capacity and affinity using molecular interaction fields and quantum mechanical procedure. The results revealed that CPO has two substrate binding sites with similar affinities for DMDBT. This finding suggests that the atypical kinetic behavior of CPO for the oxidation of DMDBT might be due to the simultaneous binding of two DMDBT molecules during its reaction cycle. Finally, we extend these results to carbazole, a nitrogen-containing PAH, through experimental and theoretical studies.

Keywords: Chloroperoxidase; Ligand docking; Sigmoidal kinetics; $\pi-\pi$ Dimers

1. Introduction

Chloroperoxidase (CPO) from *Caldariomyces fumago* is the most versatile and unusual known heme-peroxidase due to its halogenase, peroxidase, catalase and cytochrome P450-like activities exhibited *in vitro* [1,2]. This enzyme has wide potential applications, ranging from synthesis of optically pure compounds to environmentally related processes [1,3,4].

With regard to oxidative reactions, CPO behaves more like P450 cytochromes (P450) than like classical peroxidases, and bears the status of being the hybrid of two different families, peroxidases and P450s. Interestingly, both enzymes display non-Michaelis–Menten kinetics for the oxidation of polyaromatic substrates. On the one side, CYP3A4 hydroxylates pyrene with positive cooperativity due to its multiple substrate binding [5]. Indeed, substrate–substrate complexes have been detected at the cytochrome active site, evidencing a multiple substrate binding site [6,7]. On the other side, we have described a sigmoidal kinetic profile for the oxidation of 4,6 dimethyl dibenzothiophene (DMDBT) to the corresponding sulfone by CPO. This atypical kinetic performance was attributed to the binding of monomer and dimer of DMDBT (two molecules stacked mainly by $\pi-\pi$ interactions) to the active site of CPO, which leads to a cooperative kinetic behavior of the CPO [8].

In this communication, we explain the atypical sigmoidal kinetic behavior of CPO for the oxidation of DMDBT based on theoretical calculations by molecular interaction fields (molecular mechanics) and quantum mechanics procedures. First, the ligands were geometrically optimized to estimate the orbital frontiers. Then, docking studies were performed to measure the free energy of substrate binding. A dimer analog, 1,3-bis (DMDBT) propane, was used to simulate the dimer molecule inside the enzyme's active site. Finally, the binding sites were identified using the Q-SiteFinder server. Additionally, carbazole oxidation by CPO was carried out theoretically and experimentally to confirm our preliminary results with DMDBT.

*Corresponding author. Email: etorres@correo.cua.uam.mx

Molecular Simulation
ISSN 0892-7022 print/ISSN 1029-0435 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/08927020701342041

2. Methodology

2.1 Modeling

The ligands (monomer and dimer) were drawn using the Isis/draw program [9] and was converted to a three-dimensional format (i.e. pdb) using the WebLab Viewer and Molekel Visualization Package [10,11]. A geometry pre-optimization was carried out by using a HyperChem-6 software. The minimum energy structure of the ligands was obtained by means of *ab initio* (Hartree–Fock) calculations at the 6-31G** level using Gaussian 98 software [12] located in a Pentium IV computer.

To understand the recognition mechanism between CPO and the ligands, docking simulations were done on the 3-D structure of CPO from *C. fumago* (PDB code: 1CPO) [13]. Before starting the docking evaluations, the partial atomic charges (Gasteiger–Marsili formalism) as well as all rotable bonds of the ligands and the Kollman charges for all atoms in CPO were assigned by using the AutoDock Tools [14]. Moreover, missing residues were also built and hydrogen atoms were added to the amino acids of the protein with the mentioned program.

For docking studies, the latest version of AutoDock (3.0.5) was chosen because its algorithm allows full flexibility of small ligands [14]. It has been shown that it successfully reproduces many crystal structure complexes and includes an empirical evaluation of the binding free energy. The preparation of protein and ligand input structures, and the definition of the binding sites were carried out under a GRID-based procedure [15]. First, a rectangular grid box was constructed over all protein ($80 \times 80 \times 80\,\text{Å}^3$) with the grid points separated by 0.375 Å, centered on the midpoint of the ligand binding pocked (upper iron atom at 1.9 Å). The box size was decreased ($40 \times 40 \times 40\,\text{Å}^3$) to force the monomer to enter at the active site.

Previously, the CPO structure was cleaned of its water molecules and co-crystallized ligands, conserving only its heme group. All docking simulations were carried out by using the hybrid Lamarckian Genetic Algorithm, with an initial population of 100 randomly placed individuals and a maximum number of 1.0×10^7 energy evaluations. The resulting docked orientations within a root-mean square deviation of 0.5 Å were clustered together. The lowest energy cluster returned by AutoDock for each compound was used for further analysis. All other parameters were maintained at their default settings.

Finally, to determine whether two monomers could be recognized at the same time by CPO, one monomer was first docked following the conditions mentioned earlier, using the box size $40 \times 40 \times 40\,\text{Å}^3$. Then, its coordinates were pasted to CPO pdb original file, obtaining a protein–ligand complex. Finally, the second monomer was docked on this last file using a box size $80 \times 80 \times 80\,\text{Å}^3$. To identify the possible binding sites, the Q-SiteFinder software was used [16]. All protein visualizations were achieved by using a Visual Molecular Dynamics (VMD) program [17].

2.2 *In vitro* assays

The kinetic data of CPO-mediated carbazole oxidation was obtained from a substrate range of 2–70 μM in 60 mM acetate buffer, pH 3.0, [KCl] = 20 mM, $[H_2O_2] = 1$ mM using isopropanol as cosolvent (10%) at 25°C. Substrate oxidation was quantified using steady-state fluorospectroscopy with an excitation wavelength of 290 and 396 nm for emission. The enzyme concentration was maintained at 50 nM, and to assure steady-state conditions, the oxidation rates were determined under 10% substrate transformation. The obtained sigmoidal kinetic curve was fitted to the Hill equation.

3. Results and discussion

Recent progress in the computational hardware and development of efficient algorithms has assisted the routine development of molecular and quantum mechanical calculations [18]. These calculations allow the generation of test models to study and to explain some enzymatic behaviors, which are very difficult to determine experimentally at molecular detail. In this regard, 3-D computer modeling of CYP3A4 helped to determine that its active site is large enough to permit the access of two substrate molecules at the same time, which could explain the observed sigmoidal kinetics behavior [19]. In addition, molecular modeling and dynamic simulations of CPO with *cis*-β-methylstyrene and the corresponding epoxide product provided a structural and energetic basis for understanding the enantioselectivity of CPO-catalyzed epoxidation reactions [20].

It was recently reported that the DMDBT oxidation catalyzed by CPO showed a cooperative profile probably due to the capacity of the enzyme to recognize a substrate dimer (π–π dimer) [8]. Experimental evidence was given for dimer formation and its presence in the active site of CPO. In addition, it was also suggested that CPO might recognize two monomer molecules in different binding sites, although this step was not the preferred one for DMDBT oxidation. In this work, we carried out theoretical calculations of the free energy of substrate binding (ΔG) to CPO to gain specific information about the interaction of CPO with both the monomer and dimer of DMDBT. The monomers and dimers were drawn and the structures were energetically minimized before docking simulations. We employed the 1,3-bis (DMDBT) propane derivative to simulate the dimer [21].

Optimized ground-state geometry of DMDBT was obtained at the 6-31G** level. The optimized dihedral angle between the subunits of the dimer and the inter-ring bond lengths are shown in figure 1 and table 1. As can be seen, the dimer showed a non-planar structure with a dihedral angle of 136.96° and a distance of 5.1 Å between

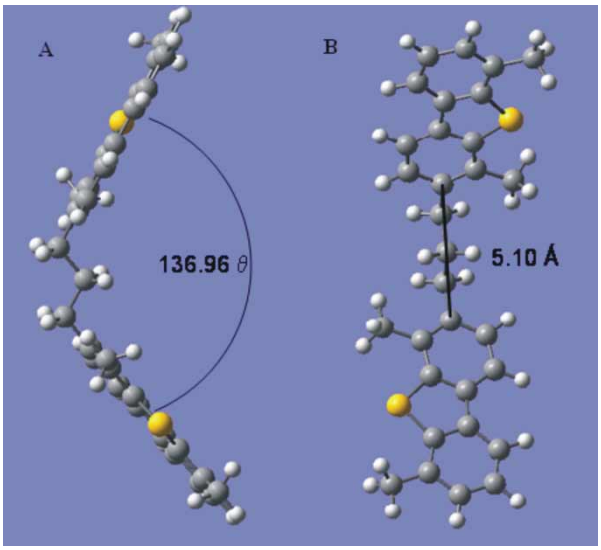

Figure 1. Angle (A) and distance in Å (B) of the optimized dimer analog, 1,3-bis (DMDBT) propane, optimized by *ab initio* (Hartree–Fock) calculations at 6-31G** level.

the α carbons to the methyl group (C7) of each DMDBT molecule. This geometry is similar to the one obtained for dimers of nitrogen-containing PAHs, thiophene, and benzene using density functional theory (DFT) calculations [22,23]. The optimized monomer and dimer were used as ligands for CPO. To this end, the Autodock 3.0 program was employed with one hundred runs of the space search to find the best complex conformation and best binding site. The docking simulation was carried out using the Metropolis method, also known as Monte Carlo simulated annealing [14]. The results obtained with the Autodock 3.0 procedure mentioned earlier are summarized in table 1. The energies of the binding event showed that CPO presented a higher affinity toward the DMDBT dimer in comparison to the monomer. These results are in agreement with the experimental results obtained previously by steady-state fluorometry [8]. The DMDBT monomer entered deep inside the active site crevice, 5 Å away of the heme iron, which is within the distances exhibited for CPO and other substrates such as phenols and sulfides, as determined elsewhere by spectroscopic techniques [24]. The DMDBT monomer interacted mainly with the residues Phe 103 and 186, Leu 70, His 105 and the catalytic residue Glu 183 (figure 2A), which are situated at the bottom of the entrance channel above the heme iron [13]. Kühnel *et al.* recently reported the crystal structure of CPO complexed with various organic substrates [25]. The authors showed that Phe 103 and 186, Val 67, His 105 and Glu 183 interacted with cyclopentadione and dimethylsulfoxide. So, Autodock predicts a binding of CPO with DMDBT similar to those found experimentally for other CPO substrates. On the other side, as can be seen in figure 2B, the dimer showed a similar alignment, with one DMDBT monomer moiety in the same position as the DMDBT monomer molecule, while the other portion fit near the entrance of the active site, interacting with Ala 267, Thr 238, and Glu 266 (figure 2B). Due to the flexibility of this region, as indicated by high temperature factors [13], there might be little steric impediment for the binding of bulky substrates, such as DMDBT dimer. Interestingly, the dimer changed its conformation in such a way that the distance between the two monomers and the angle are decreased, suggesting that a more stable configuration is adopted when the DMDBT dimer bound the enzyme (table 1). This is also in agreement with the experimental results reported earlier by our group [8], suggesting that the DMDBT dimer is more stable into the CPO active site. To analyze this point further, the sequential binding of two monomer molecules was simulated. Figure 2C shows that two molecules of monomer bound to CPO near the same positions as predicted, using the dimer analogue 1,3-bis (DMDBT) propane. The sum of energies for the sequential binding is close to the energy calculated for the dimer (table 1). However, the distance between the DMDBT monomers is shorter than the distance between the DMDBT moieties in the 1,3-bis-(DMDBT) propane molecule; 2.93 and 4.85 Å, respectively. This observation suggests that dimerization might occur inside the active site. So, it seems that CPO bears two different binding sites with almost the same affinity for DMDBT (table 1), which might allow the formation of a DMDBT dimer inside the enzyme active site. This finding could explain the sigmoidal kinetic behavior of CPO previously reported.

Based on these results, we hypothesize that the oxidation of related compounds with affinity to these two binding sites could display a non-Michaelis–Menten profile. To further analyze this point, carbazole was docked to the CPO active site using the same conditions as described earlier for DMDBT. Carbazole was chosen for its ability to form dimers in aqueous media [26], and also because it is a substrate for peroxidases [27].

A similar picture was obtained when carbazole was docked to CPO (table 1). The angle of the carbazole dimer is importantly reduced when it is docked to the CPO active site, from 138 to 66°, with a concomitant reduction

Table 1. Geometries and theoretical binding energy for DMDBT monomer and dimer optimized and docked into CPO.

Compound	*Optimized dimer distance, Å (degrees)*	*Docked dimer distance, Å (degrees)*	*Two docked monomers (Å)*	*ΔG (kcal/mol)*		
				Monomer	*Dimer*	*Second monomer*
4,6-DMDBT	5.10 (136.96)	4.85 (105.14)	2.93	−5.31	−12.36	−5.74
Carbazole	5.09 (138.01)	4.82 (66.44)	3.85	−5.65	−8.94	−6.32

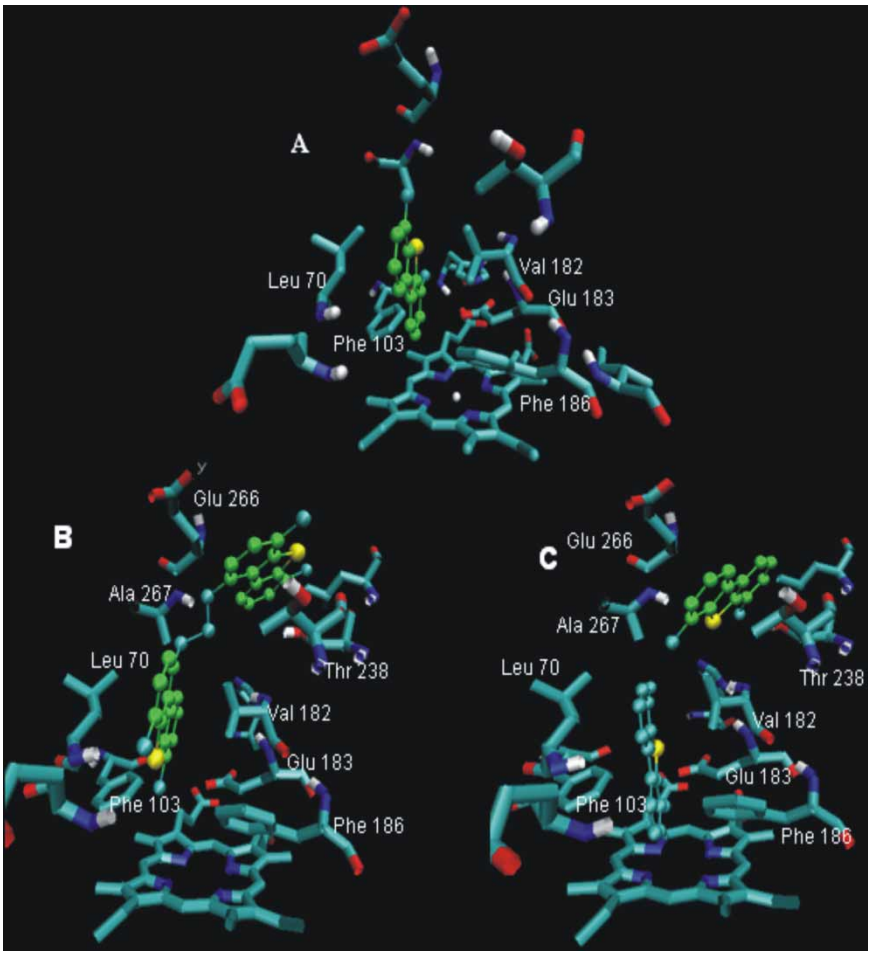

Figure 2. Energy-minimized complex formed between CPO and (a) DMDBT monomer, (b) the dimer analog, 1,3 bis (DMDBT) propane and (c) two monomers of DMDBT.

of the distance between the rings from 5.09 to 4.82 Å. This distance is even shorter when the monomers are sequentially docked to CPO, 3.85 Å (table 1). According to the binding energy, the carbazole dimer is also preferentially recognized over the monomer but with a minor ΔG when compared to DMDBT dimer. Both the binding energy and geometry data suggest that CPO could recognize the carbazole dimer as substrate. Furthermore, it seems that the carbazole dimer could also be formed inside the active site due to the reduction of the ring distance, and because this path seems to be energetically favored (table 1).

Based on these results, we evaluated experimentally the tendency of carbazole to form dimer complexes in water/isopropanol mixtures by the analysis of their emission and excitation spectra [8,28]. The emission spectra of carbazole showed a vibronic band at 356 nm corresponding to monomer fluorescence emission whereas excimer phosphorescence was observed at 450 nm, as reported elsewhere [29]. Comparing the excitation spectra of monomer and excimer species permits to unveil the presence of dimer complexes. Wavelength shift is a parameter frequently used to identify preformed ground-state complexes [30]. A $\Delta\lambda = 5$ confirms the presence of dimers for carbazole. We also found that the monomer/-excimer intensity ratio changed in the presence of CPO, indicating an interaction with both the monomer and the dimer. The intensity ratio at 356 nm decreased from 118 to 90 in the presence of CPO, which suggests a major presence of the carbazole dimer. In addition, when the enzyme is present, the peak to valley ratio of the excitation spectra for the monomer (P_M) and the dimer (P_E) are also affected. The $P_M - P_E$ value depends on the presence of preformed ground-state dimers [31] and any change may be related to the interaction with other species. In our case, the $P_M - P_E$ increased from 4.7 to 6.2 when CPO is added in catalytic concentrations (carbazole is 1000-fold more concentrated). Decrease in both the monomer/excimer intensity ratio and the $P_M - P_E$ value suggests that CPO promotes the formation of carbazole dimers, as predicted by theoretical calculations.

Since CPO may bind a monomer, two monomers, or one dimer of carbazole, we hypothesized that their presence in the active site should affect its kinetic behavior. Indeed, we found that CPO exhibited sigmoidal kinetic behavior for the oxidation of carbazole (figure 3), which the Michaelis–Menten model could not interpret. The Hill equation provides an excellent fit to the

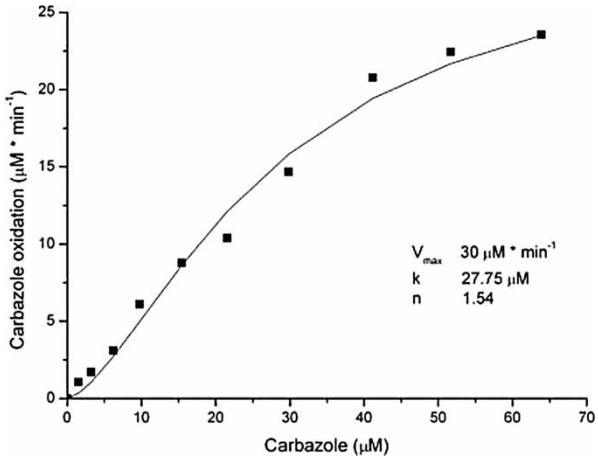

Figure 3. Kinetics for carbazole oxidation by CPO. Fitted curve was obtained using Hill equation. Conditions: isopropanol/acetate buffer pH 3, 60 mM (1:9), $[H_2O_2] = 1$ mM, [KCl] = 20 mM, [CPO] = 50 nM, 25 °C.

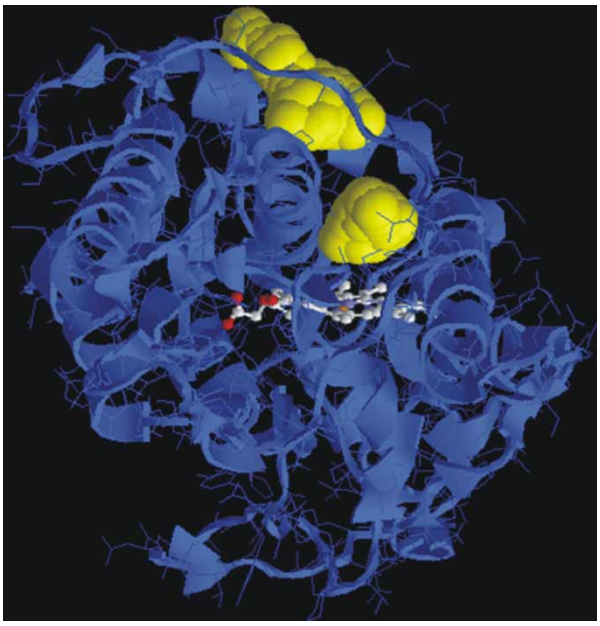

Figure 4. Binding sites 4 and 5 of CPO identified by the Q-SiteFinder program. The yellow portions represent the volumes of the binding sites found, 221 and 122 Å, respectively (Colour in online version).

experimental data. The *n* value of 1.6 does not correspond to a monomeric enzyme with a single active site such as CPO, but it might give an indication of cooperative kinetics. In principle, this could correspond to at least two substrate molecules binding to one monomeric enzyme. Some peroxidases, such as lignin, manganese and ascorbate peroxidases, show two distinct substrate-binding sites, one in the heme vicinities and another one near an amino acid residue in the protein surface [32–34]. Our preliminary results suggest that CPO bears more than one binding site for heterocyclic compounds such as DMDBT and carbazole.

Finally, we also used the Q-SiteFinder method to predict ligand-binding sites of CPO. Q-SiteFinder uses the interaction energy between the protein and a simple van der Waals probe (methyl group) to locate energetically favorable binding sites on the surface of a protein [16]. Probes with favorable interaction energies are retained and clusters of these probes are ranked according to their total interaction energies. The energetically most favorable cluster is then ranked first. The simulation produced ten different binding sites for CPO. Two of these predicted sites (ranked 5 and 6) with volumes of 122 and 153 $Å^3$ are located inside the channels connecting the heme with the protein surface. The former site, ranked 5, matched the binding site for cyclopentadione, dimethylsulfoxide and acetate [25] (figure 4); and the site ranked as 6 is one of the binding sites for iodine and bromide, as reported recently [25]. Another binding site with a volume of 221 $Å^3$ (ranked 4, energetically more favorable) was predicted at the entrance of the channel above the heme (figure 4), which coincided with the binding site found with Autodock program for one of the portions of the DMDBT dimer or for the second DMDBT monomer (figure 2C). The first three sites found by Q-SiteFinder were located at noncatalytic positions.

Thus, theoretical calculations using Q-SiteFinder also predicted that CPO has two different sites at the channel above the heme, energetically suitable for polyaromatic substrate binding; one of the sites is located inside the channel (ranked 5) and the other on the entrance of the same (ranked 4). Sites 4 and 5 suggest a pathway for substrate access to the CPO active site. Site 5 is very close to that experimentally identified recently from the crystal structure of the complexes between CPO and cyclopentadione and dimethylsulfoxide [25], and is also the binding site for DMDBT, as predicted for Autodock program. Site 4, although not yet experimentally identified, showed almost the same affinity for carbazole and DMDBT than the site ranked 5, as predicted by Autodock simulations. Due to the fact that both exhibited the same affinity, two substrate molecules could be attached to the enzyme at the same time, affecting the kinetic behavior of CPO. As a control, we carried out the docking of cyclopentadione to CPO. This substrate was recognized for CPO with high affinity at site 5 near the heme group. No binding was detected in site 6 when a second molecule of cyclopentadione was added to the docking simulations. Therefore, it is possible to suggest that CPO has two binding sites for polyaromatic compounds such as carbazole and DMDBT. The binding of two molecules of monomers or one dimer can take place in CPO, which shifts the kinetics profile from the conventional Michaelis–Menten to a sigmoidal behavior.

4. Conclusion

CPO presents atypical kinetics behavior for the oxidation of heteronuclear polyaromatic hydrocarbons determined experimentally and theoretically. In this study, we have shown that CPO can recognize as substrates different

species of one substrate, that is, monomeric, π−π dimers built either in the medium or inside the channel to its active site. Such versatile catalytic behavior may be responsible for the unusual sigmoidal kinetics that indicate kinetics cooperativity. The recognition of a π−π dimer of DMDBT and carbazole built outside and inside the active site are energetically favored against the binding of monomeric substrate. Ligand docking also showed that the π−π dimer is more stable inside the CPO's active site than when it is formed outside.

Acknowledgements

This work was supported by grants from the Mexican Petroleum Institute (D.000344 and D.000293), CONACYT and COFAA-SIP/IPN. We acknowledge Dr Marcela Ayala from IBT (UNAM) for fruitful discussions.

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