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Modeling of laccase inhibition by formetanate pesticide using theoretical approaches☆

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ABSTRACT

The inhibition of laccase enzymatic catalytic activity by formetanate hydrochloride (FMT) was investigated by cyclic voltammetry and by quantum chemical calculations based on density functional theory with a protein fragmentation approach. The cyclic voltammograms were obtained using a biosensor prepared by enzyme immobilization on gold electrodes modified with gold nanoparticles and 4-aminophenol as the target molecule. The decrease in the peak current in the presence of FMT was used to characterize the inhibition process. The calculations identified Asp206 as the most relevant moiety in the interaction of FMT with the laccase enzymatic ligand binding domain. The amino acid residue Cys453 was important, because the Cys453–FMT interaction energy was not affected by the dielectric constant, although it was not a very close residue. This study provides an overview of how FMT inhibits laccase catalytic activity.

1. Introduction

The contamination of water and food with pesticides has made the monitoring of this class of compounds a research topic of great interest in the 21st century [1,2]. Since the 1940s, the industrial production of such compounds has increased, and it continues to do so. Among several classes of pesticides, carbamate compounds are widely used insecticides and acaricides. Formetanate hydrochloride (3-dimethylaminomethyleneaminophenyl methylcarbamate hydrochloride, FMT) is applied to several agricultural crops, including grapes, mangoes, potatoes, onions, citrus fruits, beans, watermelons, peppers and tomatoes. Despite the success of FMT in pest control for the aforementioned agricultural crops, the chemical is on the priority list released by the United States Environmental Protection Agency (US EPA Regulating Pesticides) [3] because FMT is soluble in water and because the compound causes neurotoxicity due to inhibition of the enzyme acetylcholinesterase [4–7]. Therefore, analysis of the FMT residue in food and aquatic environments is an issue of great interest.

Enzymatic electrochemical biosensors are environmentally friendly, allow miniaturization and are inexpensive, and thus may be useful in the analytical detection of pesticides [8,9]. To analyze FMT and other carbamate pesticides in water and in fruits, various electrochemical biosensors have been proposed; these biosensors combine laccase enzyme (Lac) with several transductors such as composite paste electrodes with carbon ceramic [10], multi-walled carbon nanotubes [11], platinum nanoparticles-ionic liquid-graphite [12], Prussian blue film-grapheme [13], gold nanoparticle-chitosan-grapheme [14], and gold electrodes modified with gold nanoparticles (AuNPs) 15]. The rationale for these electrochemical biosensors is that carbamate compounds inhibit the enzymatic catalysis of phenolic compounds. The proposed electroanalytical procedures have been applied to the detection of carbamate pesticides in complex samples such as carrot, cucumber, lettuce, pepper, potato, grape, mango, orange, tangerine and lemon samples.

However, the electrostatic interactions between the FMT molecule and Lac amino acid residues remain unknown, which makes the pesticide's inhibition of Lac catalysis by phenolic compounds difficult to understand. Therefore, the development of a theoretical model to predict the interactions between FMT and amino acid residues in Lac could identify which amino acid residues are involved in the activity of FMT, with the aim of understanding the pesticide's inhibition mechanism and the sensitivity and stability of Lac-based biosensors.

Quantum mechanics (QM) has been applied successfully for systems smaller than 100 atoms, and QM can be expected to have a positive impact on the study of biological systems such as proteins that consist of a

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few thousand atoms [16]. QM methods have a high computational cost, but this is offset by a more realistic modeling of the electronic nature of biological systems and the wide potential applications of QM [16]. With the development of novel computational hardware technologies in the last few decades and the development and easy access to quantum chemical software programs, QM approaches such as density functional theory (DFT) [17] can be applied to more complex systems individually or in combination with classical methods [18–20]. One approach to increase the reach of QM methods is the use of fragment-based schemes such as molecular fractionation of conjugated caps (MFCC) [21] and fragment molecular orbital (FMO) [22] methods that permit accelerated calculations in these systems without losing the calculation accuracy necessary to obtain reliable results. MFCC has been successfully applied to many systems [23–25] and involves dividing large molecules such as proteins into smaller fragments to enable the calculation of properties using a quantum mechanical approach for each fragment.

Then, the results for the individual fragments can be combined to obtain the same properties for the whole system. Using all these technological advances, we have adapted a powerful tool to improve understanding of how complex systems function and, in this specific case, of how FMT interacts with and inhibits Lac catalysis of phenolic compounds, allowing the use of this protein to fabricate electrochemical biosensors for the quantification of this pesticide in samples. Because a Protein Data Bank (PDB) file for Lac cocrystallized with FMT is not available, a docking step was necessary before the DFT calculations. The docking process predicts the best conformation of the ligand for forming a stable complex within the protein pocket [26], allowing the calculation of the interaction energy by DFT. Therefore, the aim of this investigation was to propose a quantum biochemistry calculation model to investigate the electrostatic interaction between Lac and FMT to identify which amino acid residues are responsible for the interaction and the inhibition of Lac activity, thus allowing the correlation of enzyme inhibition with the concentration of FMT in real samples.

2. Experimental section

2.1. Preparation of biosensor based in laccase enzyme and electrochemical measurements

Because the inhibition of the enzymatic catalysis process is the fundamental principle underlying the use of electrochemical biosensors for the analytical determination of pesticides in fruits and water, we fabricated an electrochemical biosensor using the Lac enzyme to evaluate the FMT pesticide inhibition of enzymatic catalysis of 4-aminophenol (4-AP, phenolic substrate) using the cyclic voltammetry technique. Following a previous published procedure,[15] the Lac enzyme (*Trametes versicolor*) was immobilized on gold electrodes previously modified with gold nanoparticles, termed here Lac/AuNPs/Au. The cyclic voltammograms were recorded with a potentiostat (Autolab PGSTAT 30, Metrohm-Eco Chemie) controlled by a personal computer, using GPES version 4.9 software. Ag/AgCl/saturated KCl, graphite rod and Lac/AuNPs/Au were used as the reference, auxiliary and working electrodes, respectively. The cyclic voltammetric (CV) experiments were performed in 0.04 mol L^{-1} of Britton–Robinson (BR) buffer pH 5.0 containing 5.83×10^{-5} mol L^{-1} of the substrate in the absence and presence of FMT pesticide and at room temperature (approximately 25 °C). The data were obtained in the range from 0.0 V to 0.4 V at 10 mV s^{-1}.

2.2. Molecular docking and system optimization

Molecular docking of FMT in the catalytic site of Lac was performed using Autodock4 [27–29]. The Lac structure was obtained from crystallographic data (PDBID 1GYC), published by [30], and the FMT ion was built and optimized through density functional theory (DFT) calculations using the Gaussian03 code [30,31]. The rm062x [32] exchange-correlation functional and the 6 − 311 + G (d,p) basis set were used, and the quality of the minimization procedure was confirmed by verifying the absence of vibrational normal modes with imaginary frequencies. FMT has a pKa of 8 [33] and, given that the biosensor measurements were obtained at pH 5, the FMT should have been charged, with an extra hydrogen atom added to the nitrogen bonded to carbon 9. The planar scheme of the FMT molecular structure and its electrostatic potential surface are presented in Fig. S1.

The charge of + 1 was then considered in the calculations. During the preparation of the input files, the atomic charge of FMT was calculated using DFT methods, and Hirshfeld's charges were assigned manually. For simplification, because Autodock is unable to assign charges for copper atoms, they were removed from the input structure of the enzyme. According to the literature [30,34], the catalytic site of Lac is in a small negatively charged cavity near copper 1, as shown in Fig. S2, which presents a cartoon view of Lac with its copper ions and highlights the binding site. During the docking of FMT, a Lamarckian genetic algorithm (GA) was used. Docking was performed 10 times using the optimized structure of FMT as the input file, a GA with 25,000,000 energy evaluations per run, the population size set to 150, and a maximum of 27,000 generations per run. At the end, five hundred poses were obtained (50 poses per output) and clustered using a RMSD tolerance of 1. $Å^2$ using Autodock Tools [29,35]. A larger cluster comprising 200 poses for FMT was generated, from which the pose with larger binding energy in the most populated cluster was chosen after a visual inspection of the interaction with the heme group. The docking results using a RMSD tolerance of 1.0 $Å^2$ can be seen in Table S1.

Next, the representative pose was complexed with Lac and submitted to energy minimization steps to improve the geometry of the binding site. The Lac–FMT complex was built using the crystallographic data for Lac and the resulting representative docking pose. Then, hydrogen atoms were added to the structure, and the water molecules and the copper atoms were replaced in the output from docking, after which the whole system was optimized by the Discovery Studio (DS) program [36]. The DS approach is based on classical molecular mechanics. During the optimization, the copper ions, FMT atoms and water molecules were kept free, and most of the protein nonhydrogen atoms were kept frozen, with the exception of residue side chains near the ligand. The CHARMm force field implemented in DS, which has specific parameters for amino acids, was used to carry out the optimization procedure. The convergence threshold parameters were set at 0.001 kcal mol^{-1} $Å^{-1}$ for the maximum force per atom, 2.0×10^{-5} kcal mol^{-1} for the total energy variation, and 1.0×10^{-5} Å for the maximum atomic displacement. The structure of the Lac–FMT complex after all treatments is shown as a cartoon in Fig. S3.

2.3. MFCC scheme and energy calculations

The classically optimized structures were then used to perform the DFT simulations, adopting the dispersion correction GGA + D exchange–correlation function, an appropriate function to describe systems with dispersive forces and hydrogen bonds, as well as van der Waals interactions. The Perdew–Burke–Ernzerhof parameterization was chosen, and it was also adopted in the scheme proposed by Tkatchenko and Scheffler (TS) to model dispersive forces [37,38]. A Double Numerical Plus Polarization (DNP 4.4) basis set was chosen to expand the Kohn–Sham orbitals within an all-electron treatment scheme. A total energy variation was imposed to be smaller than 10^{-6} Ha as a threshold to achieve self-consistency, and the orbital cutoff was set to 3.7 Å. All interaction energy calculations were performed in the gas phase (vacuum dielectric constant, ϵ^0) and were repeated with various dielectric constants, assuming values of 4, 10, 20 and 40 (ϵ^4, ϵ^{10}, ϵ^{20} and ϵ^{40}, respectively), using the COSMO continuum solvation model [39].

The Molecular Fractionation with Conjugate Caps scheme (MFCC) originally proposed by [21,40] was adopted to accomplish the description of the Lac-FMT system at the quantum level [40]. To calculate the interaction energy between the FMT with Lac residues (R^i), all amino