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Designing an enzyme-based nanobiosensor using molecular modeling techniques

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Nanobiosensors can be built via functionalization of atomic force microscopy (AFM) tips with biomolecules capable of interacting with the analyte on a substrate, and the detection being performed by measuring the force between the immobilized biomolecule and the analyte. The optimization of such sensors may require multiple experiments to determine suitable experimental conditions for the immobilization and detection. In this study we employ molecular modeling techniques to assist in the design of nanobiosensors to detect herbicides. As a proof of principle, the properties of acetyl co-enzyme A carboxylase (ACC) were obtained with molecular dynamics simulations, from which the dimeric form in an aqueous solution was found to be more suitable for immobilization owing to a smaller structural fluctuation than the monomeric form. Upon solving the nonlinear Poisson–Boltzmann equation using a finite-difference procedure, we found that the active sites of ACC exhibited a positive surface potential while the remainder of the ACC surface was negatively charged. Therefore, optimized biosensors should be prepared with electrostatic adsorption of ACC onto an AFM tip functionalized with positively charged groups, leaving the active sites exposed to the analyte. The preferential orientation for the herbicides diclofop and atrazine with the ACC active site was determined by molecular docking calculations which displayed an inhibition coefficient of 0.168 M for diclofop, and 44.11 M for atrazine. This binding selectivity for the herbicide family of diclofop was confirmed by semiempirical PM6 quantum chemical calculations which revealed that ACC interacts more strongly with the herbicide diclofop than with atrazine, showing binding energies of −119.04 and +8.40 kcal mol⁻¹, respectively. The initial measurements of the proposed nanobiosensor validated the theoretical calculations and displayed high selectivity for the family of the diclofop herbicides.

1. Introduction

Many analytical techniques have been used to detect pesticides and other residues in the environment, but new prospects for detection have emerged recently with nanobiosensors,1,2 which basically comprise a biological component (e.g. enzyme, antibody) immobilized on a nanoscale detection device. Of particular importance are the nanobiosensors obtained by deposition of a receptor layer (protein) on microcantilevers with analytes detected at a concentration as low as 10⁻¹⁸ mol L⁻¹ using an atomic force microscope (AFM).3 The functionalization of AFM tips has also been exploited, with which molecular interactions can be measured with a resolution of 10⁻¹² N, thus suggesting the possible measurement of single molecule interactions using the force curve in the so-called atomic force spectroscopy (AFS).4

The fabrication of optimized nanobiosensors requires prior knowledge of various features. In addition to an adequate choice of the biomolecule to be immobilized which would interact specifically with the analyte of interest, the method of immobilization and the experimental conditions must be determined. For enzyme-based sensors, the layer-by-layer (LbL)5,6 technique has been used owing to its simplicity and versatility.7,8 For the successful deposition of the LbL film on an AFM tip, the important issues are the charge distribution over the enzyme surface and the charge of the active sites, as the latter need to be exposed to the analyte. It is clear therefore that multiple experiments need to be performed for reaching the optimized conditions, which has been the motivation for the use of molecular modeling techniques to predict the characteristics of specific systems.
In this study we investigate the properties of the enzyme acetyl co-enzyme A carboxylase (ACC), which is promising for the detection of herbicides. The family of acetyl-coenzyme A carboxylases (ACCs) have a crucial role in the metabolism of fatty acids in most living organisms.\textsuperscript{8–11} Acetyl-CoA carboxylase (ACC), in particular, catalyses the first step, namely, the carboxylation of acetyl-CoA to malonyl-CoA. This enzyme comprises a biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase (CT).\textsuperscript{12} The CT domain contains \( \sim 800 \) residues (90 kDa), which constitute the C-terminal, and corresponds to one-third of the eukaryotic domain contains Swiss-PdbViewer.\textsuperscript{17} This completed ACC structure was the initial model for the ACC dimer, whereas only the subunits of 1UYR were added using the Protein Data Bank, PDB ID: 1UYR. The missing residues in the \( x \) and \( \beta \) subunits of 1UYR were added using the Swiss-PdbViewer.\textsuperscript{17} This completed ACC structure was the initial model for the ACC dimer, whereas only the \( \alpha \) subunit was used for the initial model of the ACC monomer. All water molecules of crystallization were removed, and hydrogen atoms were added to create an all-atoms model for the Molecular Dynamics and Docking calculations. The systems simulated by Molecular Dynamics are summarized in Table 1.

<table>
<thead>
<tr>
<th>System</th>
<th>Number of solute atoms</th>
<th>Number of ions</th>
<th>Number of solvent molecules</th>
<th>Ionic strength/mol L(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC monomer</td>
<td>11 609</td>
<td>210</td>
<td>55 470</td>
<td>0.100</td>
</tr>
<tr>
<td>ACC dimer</td>
<td>23 185</td>
<td>254</td>
<td>66 782</td>
<td>0.100</td>
</tr>
</tbody>
</table>

2. Methodology

2.1 Molecular systems

The X-ray crystallographic structure of the ACC enzyme, used for the initial models, was obtained from the CT domain in the Protein Data Bank, PDB ID: 1UYR. The missing residues in the \( x \) and \( \beta \) subunits of 1UYR were added using the Swiss-PdbViewer.\textsuperscript{17} This completed ACC structure was the initial model for the ACC dimer, whereas only the \( \alpha \) subunit was used for the initial model of the ACC monomer. All water molecules of crystallization were removed, and hydrogen atoms were added to create an all-atoms model for the Molecular Dynamics and Docking calculations. The systems simulated by Molecular Dynamics are summarized in Table 1.

2.2 Molecular dynamics simulation

The modeled systems (monomer and dimer) were solvated by filling the appropriated simulation box with SPC (single point charges) water model molecules.\textsuperscript{18} Sodium and chloride ions were used to achieve the ionic strength of 100 mM for each system, which were energy minimized using 10 000 steps with the steepest descent method. After minimization the solvent was equilibrated by performing 100 ps molecular dynamics simulation at 50, 150 and 298 K, with non-hydrogen atoms positionally restrained (force constant \( 1.0 \times 10^7 \) kJ mol\(^{-1}\) nm\(^{-2}\)). Following the solvent equilibration step, for each temperature a total of 10 ns molecular dynamics simulations were performed in an isothermal-isobaric (NPT) ensemble using the leapfrog algorithm\textsuperscript{19} with a 2 fs time step. The configurations were recorded every 1 ps for analysis. The temperature was kept at 298 K by coupling the system to a Berendsen thermostat\textsuperscript{20} with a relaxation time of 0.1 ps. The pressure was maintained at 1 bar by coupling to a Berendsen barostat\textsuperscript{20} \( \text{via} \) isotropic coordinate scaling with a relaxation time of 10 ps and a compressibility of \( 4.5 \times 10^{-6} \) (kJ mol\(^{-1}\) nm\(^{-3}\))\(^{-1}\). The stretching and bending motions of the system were constrained using the LINCS algorithm.\textsuperscript{21} A 1.4 nm cutoff was used for the short-range electrostatics and van der Waals interactions. Long-range electrostatic contributions were treated \( \text{via} \) the particle mesh Ewald (PME) method.\textsuperscript{22} All simulations were carried out using the OPLS-AA\textsuperscript{23} force field within the GROMACS 4.0.4 program.\textsuperscript{24}

2.3 Electrostatic potential and solvation free energy calculations

The electrostatic potential and the hydration free energy for ACC were estimated by solving numerically the nonlinear Poisson–Boltzmann equation\textsuperscript{18,19} with the APBS (Adaptive Poisson–Boltzmann Solver) program\textsuperscript{29} in conjunction with the OPLS-AA force field parameters set.\textsuperscript{30} The structures in the aqueous solution were obtained using a dielectric constant of 78.54 for the solvent with a solvent radius of 1.4 nm, surface tension of 0.105 N m\(^{-1}\), and ionic strength of 100 mM. The internal dielectric constant of the solute was set to 2, and the apolar contribution to the solvation free energy was calculated using a surface tension coefficient of 0.105 kJ mol\(^{-1}\). The three-dimensional potentials were obtained using 129 grid points in \( x \), \( y \) and \( z \) directions.

2.4 Molecular docking

The ACC structure and the herbicides atrazine and diclofop were prepared using the ADT (AutoDockTools) program.\textsuperscript{31} The partial charges for the ligands (herbicides) were calculated using the Gasteiger–Hückel method\textsuperscript{32} implemented in the ADT program. A 3D grid was created by the AutoGrid algorithm (a subprogram of AutoDock) to evaluate the binding energies between ACC and the herbicides. Grid maps containing \( 56 \times 56 \times 126 \) points for the herbicides were used to constrain them within the active sites of ACC. The Lamarckian genetic algorithm (LGA) was applied to search the conformational space of the herbicides, while keeping the ACC structure rigid. For each run a set containing ten LGA docked structures was obtained, from which two clusters of docked...
structures were chosen based on two criteria: (i) lowest $E_{\text{Total}}$ values; and (ii) clusters within a root mean square deviation (RMSD) limit of 2.0 Å.

2.5 Quantum chemical calculations

In order to evaluate the binding energy differences between diclofop and atrazine herbicides, single point quantum chemical calculations were performed for the ACC active site cavity model, the herbicide and the complex (ACC active site cavity + herbicides). The calculations were performed using the PM6 semiempirical Hamiltonian implemented in the MOPAC2009 software package.\(^{33}\) In order to build the ACC active site cavity model, all the residues within 10.0 Å of the cavity were retained and all the others were deleted. The remaining binding was completed by adding hydrogen atoms. Thus, the binding energy was calculated by subtracting the heat of formation of the ACC active site cavity and the herbicide from the complex (ACC active site cavity + herbicide).

2.6 Atomic force microscopy

A Nanoscope V (VEECO) SPM instrument was used to characterize the enzymes and herbicides film surfaces. AFM has the advantage of providing images of polymer and organic materials coated on insulating substrates, which prompted us to choose it as a characterization method for studies involving protein films. The enzyme was immobilized onto a thiol self-assembled monolayer (SAM) formed on the gold-coated AFM tip. For quantitative force experiments the geometry of the tip and spring constant, $k_c$, of the cantilever need to be known. Several procedures have been applied to characterize the size and shape of tips and cantilevers.\(^{38}\)

3. Results and discussion

The biosensor under design requires the immobilization of ACC on an AFM tip to detect enzyme-inhibiting herbicides, such as diclofop. According to the literature,\(^9\) ACC may exist in solution as a monomer and a dimer, both of which have catalytic activity, suggesting that dimerization is not an absolute requirement for the catalytic activity. Experimental studies showed that the dimer form is the wild-type enzyme and specific mutations on its interface are responsible for inducing monomeric behavior in solution, whose catalytic activity was weaker than that observed for the wild-type enzyme. It is therefore necessary to know the intrinsic characteristics of the monomer and dimer of ACC in solution to select the suitable enzymatic structure (monomer or dimer) for interaction with the pesticide. This interaction is strongly related to the conformational fluctuation of the enzyme in solution, from which one infers that the structural dynamics of ACC must be determined for an optimized design of the nanobiosensor.

The structural dynamics of ACC was evaluated by computing its root mean square displacement (RMSD) during the molecular dynamics simulation, in relation to its initial structure. Fig. 1 shows clearly the difference in the structural fluctuation between the monomer and dimer in an aqueous solution. The monomer completely loses its initial structure, and structural equilibrium is not achieved even after 9 ns of molecular dynamics simulation. The lability of this monomeric structure suggests that interaction forces between ACC and an herbicide—at the reactive site—can be weakened by these conformational changes, resulting in poor force propagation and inaccurate detection. In contrast, the ACC dimer exhibited only 0.3 nm of conformational fluctuation, showing a relative structural rigidity in the aqueous solution with equilibrium being reached after only 3 ns. One then infers that the interaction force between the dimeric ACC and a pesticide at the active sites is more likely to be transmitted to the AFM tip due to the rigidity of this wild-type enzyme, which avoids absorption of the greatest part of mechanical perturbation produced by pesticide–ACC interactions.

The structural fluctuations and the differences between the monomer and dimer of ACC can be attributed to two main factors. The first is the electrostatic interaction between charged amino acids such as arginine (ARG), lysine (LYS), aspartic acid (ASP) and glutamic acid (GLU). The second is the hydration of the polar amino acids, increasing the protein mobility.

To evaluate the electrostatic repulsion between charged groups with the same signal, the total translation of the amino acids ARG, LYS, ASP and GLU was calculated from the entire molecular dynamics trajectory. This calculation revealed that amino acid groups with like charges tend to stay far from each other. As a consequence, the variation in distance was 0.56 nm, at least 7% greater than the average mean distance variation of all the atoms in ACC. However, electrostatic attraction between oppositely charged amino acids leads to salt bridges that stabilize the initial structure of both monomeric and dimeric enzyme structures. The presence of salt bridges was assumed only when the distance between ARG and ASP, ARG and GLU, LYS and ASP, and LYS and GLU was less than 0.32 nm. Table 2 displays the number of salt bridges in the beginning and at the end (10 ns) of the molecular dynamics simulation. One notes that the number of salt bridges in the dimer is more than double that in the monomer. Therefore, the electrostatic interaction among oppositely charged amino acids, e.g. ARG, LYS, ASP and GLU, on the border...
between the two monomers in the dimer yielded the small structural fluctuations shown in Fig. 1, with a rigid structure in the aqueous solution. Nevertheless, the number of salt bridges decreases during the simulation (see Table 2), which should be ascribed mainly to the interaction between water molecules and these charged amino acids. This solvation effect is best illustrated by analyzing the number of hydrogen bonds between water molecules and ACC given in Table 2.

It is clear from Table 2 that the number of salt bridges is inversely related to the number of hydrogen bonds as a result of water solvation of these hydrophilic groups. The charged amino acids induce new hydrogen bonds between water molecules and ACC, thus decreasing the number of salt bridges and leading to the structural fluctuation in the enzyme structure. The intrinsic feature of the ACC monomer in having more accessible area for the solvent per atom than the dimer implies that a larger number of charged amino acids are exposed to water molecules under thermal motion. Therefore, the solvation effect seems to be the main factor for the fluctuation.

To further describe the solvation effect on the structural fluctuation and the structure stabilization of ACC by the water molecules, the electrostatics and apolar contributions to the free energy of solvation of the ACC monomer and dimer were calculated via Poisson–Boltzmann electrostatics using the APBS program.34 The initial structure (crystallographic) and the final structure, from the last 10 ns of simulation, were selected and calculated with the same ionic strength, so that the conformational fluctuation was the only variable. There are no experimental values in the literature to compare with the calculated solvation free energy. These calculations can be potentially used to predict relative solvation stabilization for the different forms of the ACC enzyme. According to the results given in Table 3, the solvation free energy is mainly governed by the electrostatic contribution, which is consistent with the relatively large number of charged amino acids in the enzyme structure. The increase (in modulus) in the electrostatic contributions, from 0 to 10 ns, of 1680 kJ mol$^{-1}$ and 3500 kJ mol$^{-1}$ for the monomer and dimer, respectively, explains the increase in the number of water molecules hydrogen bonded to the charged groups during the molecular dynamics simulation. On the other hand, the apolar contribution varied only slightly during the simulation, viz. 20 and 140 kJ mol$^{-1}$ for the monomeric and the dimeric structures, respectively.

The total free energy of solvation given in Table 3 confirmed that the relaxed enzymatic structure is energetically stabilized in an aqueous solution, reinforcing the hypothesis that solvation effects are important for the structural fluctuations. This suggests that the exposed charges on the ACC surface are strongly solvated by water molecules that diffused within the protein structure, reducing the direct electrostatic repulsion among like charged groups, and inducing conformational changes in the protein structure.

The results presented so far confirmed that the dimeric structure is the most probable for ACC in aqueous solutions, and to be immobilized on an AFM tip to produce a nanobiosensor and detect herbicides. The next step is to decide how the immobilization should take place, i.e. which would be the best orientation to adsorb on the AFM tip while leaving the active sites exposed to the solvent in contact with the tip. Because adsorption with electrostatic interactions between oppositely charged molecules is one of the main techniques used for biosensing,35,36 the electrostatic potential of ACC was calculated by solving the Poisson–Boltzmann equation using the APBS program34 (see Methodology). The electrostatic landscape shown in Fig. 2 indicates that ACC has positively and negatively charged groups located at distinct, specific regions on its surface due to the presence of charged amino acids such as ARG, LYS, ASP and GLU. According to the crystallographic data for ACC complexed with the herbicide diclofop,13 and the electrostatic potential, the positive charges are mainly located close to the active sites, which present high percentage of the positively charged amino acid: LYS. This suggests that AFM tips functionalized by negatively charged functional groups (R–COO$^-$ or SiO$_2$ tips) are not suitable to immobilize ACC. On the other hand, the negative groups (ASP and GLU) far from the active sites shown in Fig. 2

### Table 2 Number of salt bridges in ACC and number of water molecules making hydrogen bonds with the enzyme

<table>
<thead>
<tr>
<th>System</th>
<th>Time</th>
<th>Number of salt bridges</th>
<th>Number of hydrogen bonds between ACC and water molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>0 ns</td>
<td>41</td>
<td>1471</td>
</tr>
<tr>
<td></td>
<td>10 ns</td>
<td>35</td>
<td>1675</td>
</tr>
<tr>
<td>Dimer</td>
<td>0 ns</td>
<td>85</td>
<td>2797</td>
</tr>
<tr>
<td></td>
<td>10 ns</td>
<td>78</td>
<td>3134</td>
</tr>
</tbody>
</table>

### Table 3 Free energy of solvation (kJ mol$^{-1}$) for the monomer and dimer of ACC enzyme at the beginning and after 10 ns of molecular dynamics simulation

<table>
<thead>
<tr>
<th>System</th>
<th>$\Delta G_{solv(\text{electrostatic})}$</th>
<th>$\Delta G_{solv(\text{apolar})}$</th>
<th>$\Delta G_{solv(\text{total})}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>$-2.73 \times 10^4$</td>
<td>$3.94 \times 10^4$</td>
<td>$-2.34 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>$-2.90 \times 10^4$</td>
<td>$3.96 \times 10^4$</td>
<td>$-2.50 \times 10^4$</td>
</tr>
<tr>
<td>Dimer</td>
<td>$-4.63 \times 10^4$</td>
<td>$6.52 \times 10^3$</td>
<td>$-3.98 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>$-4.98 \times 10^4$</td>
<td>$6.66 \times 10^3$</td>
<td>$-4.31 \times 10^4$</td>
</tr>
</tbody>
</table>

Fig. 2 Electrostatic potential ($-5k_BT/e$ to $+5k_BT/e$) of the ACC enzyme, which shows the most negative potential as the probable immobilization area.
also suggest that AFM tips functionalized with positively charged groups, such as R–NH$_3^+$ and aminopropylethoxysilanes, would unblock the active sites, leaving them free to interact with the herbicide.

On the basis of the electrostatic potential for the ACC surface and assuming an AFM tip functionalized with positively charged groups, it is possible to suggest the possible orientation of the enzymes on the tip, as displayed in Fig. 3. In the latter, many ACC enzymes (each one shown in a different color) are adsorbed on the functionalized surface of the AFM tip.

The interaction between the ACC dimer and herbicides can be calculated using molecular docking, whose use is justified by the small structural fluctuation of the ACC dimer shown in Fig. 1. The two herbicides used were atrazine and diclofop shown on the top of Fig. 5, which were made to dock to the active site of ACC. The docking calculations resulted in 10 possible conformations for each herbicide in the active site. From the docked herbicides on the ACC active site, the most favorable clusters were used to calculate the binding energy using the semiempirical PM6 Hamiltonian implemented in the MOPAC2009 program. Binding energies of $-119.04$ and $+8.40$ kcal mol$^{-1}$ for diclofop and atrazine, respectively, were found. These results are consistent with the experimental finding that diclofop belongs to a specific class of inhibitors for ACC, thus exhibiting a more favorable binding energy than atrazine.$^{13}$ The positive binding energy for atrazine is expected, since it is not known as an inhibitor of ACC, but an inhibitor of phosphodiesterase.$^{37}$ These theoretical predictions have been confirmed with preliminary results for the proposed nanobiosensor, as indicated in Fig. 4. The average interaction force between the diclofop and ACC enzyme is ca. 3 times the force between atrazine and protein.

The perfect binding of diclofop to the ACC active sites is illustrated in Fig. 5, obtained from the conformation of the

![Fig. 3](image-url) Artwork of the enzymatic nanobiosensor. (A) Schematic representation of the detecting principle for the nanobiosensor. The ACC molecules were adsorbed on the AFM tip from a solution owing to electrostatic attraction. The functionalized tip was then immersed in a liquid cell containing a solid support coated with a layer of a herbicide. The interaction between ACC and the herbicide was obtained by measuring the force curve as the AFM tip approached the herbicide-containing sample and was later withdrawn. (B) Various immobilized ACC molecules oriented on the surface of a functionalized AFM tip. The change in color is just to facilitate visualization.

![Fig. 4](image-url) Results for the adhesion forces obtained from the nanobiosensor suggested by the molecular modeling techniques. These forces were obtained by taking the force curves in an atomic force microscope Nanoscope V (Veeco).

![Fig. 5](image-url) Two diclofop herbicides efficiently docked to the ACC active sites. The inset shows the structures of the herbicides (A) atrazine and (B) diclofop.
 energetic most favorable cluster. In such a conformation a favorable electrostatic interaction is observed between the carbonyl group of the herbicide and the positively charged groups in the active site, which may explain the small RMSD for diclofop in Table 4. The analysis of Table 4 also reveals that atrazine has a large RMSD implying a relatively high mobility inside the active site. Therefore, the molecular modeling techniques used in this work confirmed that the atrazine herbicide is not suitable for inhibition of ACC.

The docking calculations showed that the inhibition coefficient, \(K_i\), i.e. the concentration of the herbicide required to inhibit the ACC activity, was much lower for diclofop, as indicated in Table 4. The authors acknowledge CAPES, CNPq, FAPESP and FAPEMIG for the financial support.

4. Conclusion

The results presented here demonstrated that molecular modeling techniques are useful for predicting structural and electrical properties of biomolecules that could be used in nanobiosensors. Since the biomolecules normally employed are very large, one cannot rely entirely on \textit{ab initio} quantum methods and has to resort to classical and semiempirical methods. Molecular dynamics, for instance, was key to showing that the dimeric form or wild-type ACC is structurally stable in aqueous solution, which then allows one to predict how immobilization on an AFM tip should be performed for a nanobiosensor. The surface charge distribution of ACC, obtained by solving the Poisson–Boltzmann equation, indicated that the enzyme should be immobilized on a positively charged tip, so that the positive active sites of ACC would be exposed to the analyte.

Perhaps one of the most important results was the identification of the type of herbicide best suited for biosensors incorporating ACC. With a combination of molecular docking, molecular dynamics simulations and semiempirical quantum chemistry, we could show that ACC interacts preferentially with diclofop—in comparison to atrazine. Moreover, the calculations allowed us to identify the reasons why ACC-based biosensors should be selective for the family of diclofop herbicides. Indeed, preliminary data from force curves confirmed this selectivity, thus validating the calculations presented here. In summary, the use of molecular modeling methods may save time and efforts by predicting optimized conditions for the fabrication of nanobiosensors, which eliminates the need for performing a number of time-consuming experiments.

Acknowledgements

The authors acknowledge CAPES, CNPq, FAPESP and FAPEMIG for the financial support.

Table 4 Docking results for the atrazine and diclofop herbicides

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atrazine</th>
<th>Diclofop</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMSD/Å</td>
<td>11.769</td>
<td>0.800</td>
</tr>
<tr>
<td>(K/\mu\text{M})</td>
<td>44.110</td>
<td>0.168</td>
</tr>
</tbody>
</table>

References