STRUCTURAL AND ELECTRONIC PROPERTIES OF NBPT INHIBITOR ATTACHED TO UREASE

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Abstract. In this study, the structural and electronic properties of the N-(n-Butyl) Thiophosphoric Triamide (NBPT) inhibitor, in the form of monoamidothiophosphoric acid (MATP), as attached to urease enzyme, has been investigated. These include the electron density, molecular orbitals involved in the interactions, and the whole system's charge distributions. The difference between the interaction of urease-NBPT and urease-urea was conducted throughout this study. This comparison was crucial to prove the NBPT inhibitor's mechanism to slow down urea's hydrolysis in the soil solution. The quantum mechanical calculations were performed at the level theory B3LYP/6-31G(d,p). The urease-NBPT complex has higher interaction energy than the urease-urea complex, in which the interaction energy is –1.6787 eV. The urease-NBPT complex has a lower molecular electronic energy gap than the urease-urea complex, at 0.9527 eV. The graphical representation of HOMO, LUMO, and electrostatic potential maps indicates that the NBPT inhibitor can create favourable interaction with the atoms at urease's active site.

1. Introduction
In the agriculture industry, urea is the most widely used form of fertilizer. This is due to its high nitrogen (N) content with relatively low production costs. Nitrogen is a necessary nutrient for plant growth and must be diffused to the plant area's soil solution. However, urea will undergo catalytic hydrolysis by the urease enzyme after its application to the soil. This process will lead to several adverse events, such as the volatilization of ammonia gas, increasing the soil's pH, and the accumulation of nitrogen dioxide. Several studies have been conducted to prevent these phenomena. One of them is by applying an inhibitor to reduce the rate of the urea hydrolysis process [1].

Commonly, the inhibitor acts as a competitor to the urea in the catalytic process, by binding to the enzyme structure before urea can do so. As a result, the urea molecule will diffuse easily into the soil solution. This will increase the percentage of N uptake to the soil solution and reduce the volatilization of the ammonia gas to the surrounding. Several inhibitors have been identified, but the most successful and commercial one is N-(n-Butyl) Thiophosphoric Triamide (NBPT) [2]. The mechanism of the NBPT inhibitor is unique. NBPT inhibitor is not the direct inhibitor, and it must be converted into its intermediate diamido derivative (known as NBPD) which replaces one of the amide groups with oxygen [3]. The structure breakdown into monoamidothiophosphoric acid (MATP) with the loss of n-
butyl amine, and the oxygen from the MATP structure forms a bond with the two nickel ions at the active site of the urease, creating a tridentate nature of bond [3]. These bonds will reduce the probability of the urea to bind with the urease enzyme.

In this study, the interaction between NBPT inhibitor-urease complex, as shown in Figure 1, will be studied as a comparison with the interaction of the urease-urea. Even though NBPT is a well-known inhibitor in the agriculture industry, the explanation of the NBPT inhibitor-urease complex and its electronic properties including stability, energy and the binding site remains unclear. The microscopic view is needed to complement the available crystallographic data of the NBPT inhibitor-urease complex. Theoretical investigations are thus needed as the microscopic properties are difficult to obtain through experiments.

Figure 1. Structure of NBPT inhibitor in the region near the active sites of the urease enzyme. Only the crucial parts are represented using the rods and ball and stick. The rest of the irrelevant parts are in ribbon-shaped structure (brown colour). The highlighted part is enlarged to show in detail the atoms involved. The labels used are explained in the text.
2. Methodology

In this theoretical study, the G09 suite of programs [4] was used to perform the quantum mechanical calculations at the level of B3LYP/6-31G(d,p). This method of B3LYP and the basis set of 6-31g(d,p) are the standard in the density-based calculations for molecular systems [5]. Since the hydrogen interactions are essential in the combination of the NBPT inhibitor and urease, the corrections from the empirical dispersion of Grimme will be invoked [6]. The inclusion of this empirical correction enables efficient corrections to the weak interactions between components in a system [7].

Previous reports indicate that the active sites of urease are responsible for holding the NBPT inhibitor [8]. Thus, the investigations of the most stable structure of NBPT inhibitor attached to the urease have been focused on the region around the active site of urease. To use the whole structure of urease in the simulation at the intended level of theory would require enormous computing power. A common method to overcome the lack of computing power while retaining a certain level of accuracy is by using the cluster approach. By using this approach, a model cluster has been chosen to represent the urease adequately. The active site of urease was included. The cluster that was used in the present study is shown in Figure 1.

In the model, the two nickels are the active sites of the urease, represented in green spheres in Figure 1. The model is the result of truncating the non-relevant parts of the urease in the interactions between inhibitor. The residues that remain consist of aspartate (Asp), histidine (His), and lysine (Lys). The practice of modeling the cluster structure of the urease has been reported in the study of Benini et al. [9] and Mazzei et al. [3].

Instead of the whole structure of NBPT inhibitor, in this study, only MATP, as depicted in Figure 2, was assigned at an initial region near to the nickel atoms. This is due to the fact that MATP is responsible for binding at the active sites of the urease. Geometry optimizations were performed to obtain the most energetically stable structure. The interaction energy $E_{\text{interaction}}$ can be found by using the formula:

$$E_{\text{interaction}} = E(\text{system}) - E(\text{urease}) - E(\text{molecule})$$  \hspace{1cm} (1)

Where $E(\text{system})$ is the energy of the system, $E(\text{urease})$ the energy of urease, and $E(\text{molecule})$ is the energy of MATP (and the energy of urea for the case of urease-urea).
Figure 2. Configuration of the MATP, the structure derived from NBPT that is responsible for binding at the active sites of urease. The numbers after the chemical symbols (i.e. H(hydrogen), N(nitrogen), P(phosphorus), C(carbon), S(sulphur)) are aids to identify the positions of the atoms of the structure.

As a comparison to the strength of the MATP-urease interaction, the same procedure of finding the interaction energy will be repeated for urea attached to the urease.

With the availability of the most stable structure of the MATP attached to urease, the structural, stability and the electronic properties can be obtained, and are represented graphically through post-processing the wavefunction. This includes the electron density, molecular orbitals, and the electron distributions of the whole system.

3. Results and discussion

3.1 Interaction energies

The interaction energies obtained using Equation (1) are tabulated in Table 1. Using Equation (1), the more negative the $E_{\text{interaction}}$, the stronger the interaction is. It can be seen that, the urease-MATP structure has higher interaction energy compared to the urease-urea structure (−1.6787 eV versus −1.3237 eV). This urease-MATP structure also has higher interaction energy than the reported values for barbituric acid (−0.0707 eV [10]), AHA (−0.1809 eV [11]) and thiourea inhibitors (−0.2193 eV [11]). The higher interaction energy means that it needs more energy to break the interaction; hence the MATP will bind more strongly to the active sites of urease as compared to urea.

Table 1. The interaction energies of the complexes in this study. Unless otherwise specified, the values of the energies are in Hartree.

| Complex               | $E_{\text{system}}$  | $E_{\text{urease}}$ | $E_{\text{molecule}}$ | $E_{\text{interaction}}$ (eV) |
|-----------------------|----------------------|---------------------|-----------------------|-------------------------------|
| Urease-urea           | −4960.6063           | −4705.3732          | −225.1844             | −1.3237                       |
| Urease-MATP           | −5577.2531           | −4705.2985          | −871.8929             | −1.6787                       |

3.2 The inter-atomic distance between the atoms of MATP and urea attached to the urease enzyme

From the optimized geometry of the complexes, the distances between selected atoms of the cluster structures of MATP and urea near the active site of urease were measured. The objective is to gauge the similarity between the two complexes. The selected distances of atoms in the structure of MATP at the active site of the urease are shown in Figure 3(a). The selected distances were picked from the atoms that bond with the nickel ions only as the interaction of MATP and urease occur in that region. In Figure 3(b), the selected distances of atoms in the cluster structure of urea at the active site of the urease are given. It can be seen that the distances considered for urease-urea are shorter than urease-MATP, except the one involving nickel atom with O6 (Lys-220). The shorter inter-atomic distance is related to the stronger interaction between the constituent atoms; hence the results seem to indicate that urease-urea has stronger interaction than urease-MATP. However, from another perspective, the considered distances of urease-MATP can be seen to be comparable with those of urease-urea (with the difference between 0.02 and 0.31 Å). Coupling this with the energetic point of view (as discussed in Section 3.1), the MATP can still fulfil its purpose to inhibit urease in the soil solution. The selected inter-atomic distance for urease-MATP also showed similar values with the previous literature [3].
3.3 Molecular orbital analysis

The graphical contour of highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of urease-MATP and urease-urea are shown in Figures 4 and 5. HOMO of urease-MATP, as depicted in Figure 4(a), is mainly accumulated in the area of nickel ions bonded with MATP. In contrast, LOMO (Figure 4(b)) is gathered at the site of the nickel ions bonded with the aspartate residue (Asp-363) and histidine residue (His-275). From this observation, it can be concluded that there is a chemical reactivity that occurred between nickel ions with the MATP moiety, aspartate residue (Asp-363) and histidine residue (His-275). As shown in Figure 5(a) for the urease-urea complex, HOMO is also detected in the area of nickel ions with urea. In contrast, LOMO (Figure 5(b)) is seen at the aspartate residue (Asp-363) and histidine residue (His-139). Thus, it can also be concluded that there was a chemical reactivity between nickel ions with the urea, aspartate residue (Asp-363) and histidine residue (His-139).
Figure 4. (a) HOMO and (b) LUMO of Urease-MATP complex.
Figure 5. (a) HOMO and (b) LUMO of the urease-urea complex.

The molecular electronic gaps, obtained as the difference between HOMO and LUMO, are tabulated in Table 2. From Table 2, the gap energy of the urease-MATP complex is lower than the urease-urea complex. The low energy gap indicated that the electrons from the HOMO of the urease-MATP complex are easier to be excited to LUMO, compared to the urease-urea complex.

Table 2. Molecular electronic gaps of the complexes considered in this study. Unless otherwise specified, the values of the energies are in Hartree.

| Complexes     | HOMO   | LUMO   | Energy gap (eV) |
|---------------|--------|--------|-----------------|
| Urease-MATP   | -0.02606 | 0.00895 | 0.9527          |
| Urease-urea   | -0.06907 | 0.02082 | 2.2256          |
3.4 Electrostatic potential analysis

An electrostatic potential map presents the value of the electrostatic potential at locations on a density surface. It can be used to indicate the area for the electrophilic and nucleophilic attack as the reactivity of the molecule [12]. Red regions show excess negative charge; blue areas show excess positive charge and the green areas show the charge is neutral [13].

The graphical representation of the electrostatic potential map is shown in Figure 6 for urease-MATP and Figure 7 for urease-urea. For urease-MATP, the red region is observed at the aspartate residue, the blue region is observed at the histidine residues, and the green residue was observed at the rest of the urease-MATP structure. It can thus be concluded that the aspartate residue has an excess of a negative charge, making it suitable for the electrophilic attack. In contrast, histidine residues have an excess of a positive charge, making it ideal for the nucleophilic attack. However, the green regions at the interaction of MATP with the active site of the urease indicate that it has achieved neutral charge distribution. As can be seen in Figure 7, the red region is observed at the aspartate residue, the blue region is observed at the histidine residues, and the green residue is observed at the rest of the urease-urea structure. Hence, the aspartate residue also has an excess of a negative charge, while histidine residues also have an excess of a positive charge. Similar to the urease-MATP complex, the green regions at the interaction of urea with the active site of the urease indicated that it had achieved neutral charge distribution.

Figure 6. The electrostatic potential map of the urease-MATP complex.
Figure 7. The electrostatic potential map of the urease-urea complex.

4. Conclusion
In this report, the study of the NBPT inhibitor (in the guise of MATP moiety) present at the active site of the urease has been conducted. The urease-MATP complex is found to be more stable compared to the urease-urea complex and has interaction energy of $-1.6787\, \text{eV}$. The inter-atomic distances for urease-MATP are comparable with those of urease-urea, thus showing that NBPT can inhibit urease in the soil solution without much alterations to the structure of urease. The structure of NBPT inhibitor with urease showed that it has smaller molecular electronic gap compared to the structure of urea with urease. The HOMO, LUMO, and electrostatic potential maps indicate there are favourable interactions of MATP at the active site of the urease.

Overall, this study is crucial in providing the microscopic view of NBPT, especially MATP at the active site of urease, as a complement to the crystallographic structure from the previous experimental work. This study will also benefit the agriculture sector, specifically on improving the soil fertilization efficiency.

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