

Using Electronic Theory to Examine Horseradish Peroxidase-Mediated Coupling of Estradiol

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Abstract

This research studied the oxidative polymerization of estradiol using molecular docking and electronic theory. Horseradish peroxidase removes estradiol quickly but it does not react as readily with the dimers. The structural identities of the dimers indicated that the oxidative coupling reactions take place at carbon atoms with high electron density. This research is the first to reach this conclusion. The electrons shift after the coupling reaction, and some carbon atoms actually increase in electron density, even though the reaction is an oxidation. This research adds more momentum to the emerging area of electron density-based modeling for proactive discovery of environmental byproducts.

Keywords: Oxidative coupling; Frontier electron density; Estrogens; Wastewater; Horseradish peroxidase

Introduction

Phenolic steroids can be removed from wastewater by oxidatively polymerization with horseradish peroxidase (HRP) [1-5]. Oxidative coupling (OXC) is fast and produces insoluble particulates that can be easily removed during sedimentation or filtration. The HR-OXC catalytic cycle involves: 1) a hydrogen peroxide-induced transfer of two electrons from the iron (III) residue in the active site of HRP, 2) a one-electron reduction in which a phenolic substrate donates an electron to the HRP iron (IV)⁺ residue, 3) a second one-electron reduction in which a phenolic substrate donates an electron to the HRP iron (IV) residue, and 4) reaction between the two phenoxy radicals, producing a dimer. OXC is not as energy intensive as other advanced oxidation processes (such as ozonation) and it now stands as a promising and potentially sustainable option for addressing the presence of phenolic chemicals (including some endocrine disruptors) in water. However, OXC is not well understood fundamentally, and there is not yet any established method for predicting how dimers will be formed from the parent phenolic structures.

Recent research has demonstrated that the oxidation of phenolics can be better understood using frontier electron density (FED). Electron density calculations can elucidate the fundamental principles governing reactivity by predicting which positions on the molecule will most likely undergo electrophilic attack. The general concept is that an electron-poor molecule will readily attack a position of large electron density. FED has been studied for many years, and many researchers have demonstrated that FED is fundamentally connected to chemical reactivity [6-9].

Most recently Harper [10] successfully predicted where ethinyl estradiol would be degraded by first determining the electron distribution. Yi [11] carried out a similar study with trimethoprim. This recent work is now showing that biologically-mediated electron transfer reactions can be understood using FED-based approaches and this means that we are close to being able to carry out a priori predictions concerning how steroidal compounds will be transformed in the environment. The FED-based approach is relevant to oxidative coupling (OXC) and it may help predict the structure of polymers present in water and improve the understanding of OXC. In order to test this theory, the current work aims to apply frontier electron density (FED) theory to explore the OXC of estradiol (E2), a phenolic steroid of great interest in the water quality community.

Materials and Methods

Overview

Estradiol was oxidatively polymerized in batch experiments and the binding of HRP with estradiol was simulated to determine binding characteristics. Dimer byproducts were obtained from literature, and the electron density of estradiol and these dimer structures was determined. Finally, we restricted the scope of this work to the step where estradiol is converted into dimers; the reason for this is because higher order polymers are more insoluble and their structures are not well known.

Materials

The following materials were purchased from Sigma-Aldrich (St. Louis, MO): phenol (CAS 108-95-2), estradiol, E₂ (CAS 50-28-2), hydrogen peroxide (50 wt%, CAS 7722-84-1), extracellular horseradish peroxidase (type I, R_z=1.3), polyethylene glycol (CAS 25322-68-3), 4-aminoantipyrine (AAP) (CAS 83-07-8), reagent-grade acetonitrile (CAS 75-05-8), and methanol (CAS 67-56-1).

Enzyme activity assay

A colorimetric assay was used to measure the HRP activity and concentration. The enzyme activity is proportional to the production rate of a constituent that absorbs light at a peak wavelength of 510 nm and with an extinction coefficient (ϵ) of 7100 M/cm. The assay mixture consisted of 10 mM E2, 2.4 mM AAP, and 0.2 mM H₂O₂. One unit of activity (U) was defined as the number of micromoles of hydrogen peroxide utilized per minute at pH 7.4 and 25°C [12]. Absorbance was monitored at 510 nm with a UV/VIS spectrophotometer (Spectronic 20, Bausch and Lomb) every 5 seconds for 1 minute following a reaction

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initiation. All assays were performed in triplicate. Relative standard deviations (RSD) of triplicate measurements were always less than 5%.

HPLC analysis of phenolic substrates

Estradiol concentrations were measured with an Agilent 1200 series high-performance liquid chromatograph (HPLC) equipped with an Eclipse XDB-C18 column (15 × 4.6 mm, 5 μm particle size) [3]. The concentrations were determined with UV absorbance (wavelength=197 nm) with external calibration. The mobile phase consisted of 40% reagent-grade acetonitrile (ACN) and 60% deionized water (DI). The flow rate was 1.0 ml/min. The retention time for estradiol was 7.27 min.

Electron density analysis

Frontier electron density (FED) analyses were performed to determine the electron density profile for E₂ and for relevant dimer products. The Unrestricted Hartree-Fock (UHF) method and STO-3G basis set were employed for initial structure optimizations using the program Gaussian 0327. UHF/6-31G(d) calculations were used for final geometry optimizations, computing vibrational frequencies, and in calculating the electron density of each compound. The FED for all carbon atoms were computed using the following equation:

$$f_r = [2 \times \sum_i (C_{ri} \text{HOMO})^2] \text{ (Equation 1)}$$

For an electrophilic reaction, the highest occupied molecular orbital (HOMO) densities are normalized by the energy of the frontier molecular orbitals at ground state. The coefficient of each atomic orbital, C_{ri}, is used to produce the frontier movement, where r is the number of carbon atoms in i: 2s, 2px, 2py, and 2pz orbitals. All simulations were performed on computers located at the Pittsburgh Supercomputer Center. The E_{HOMO} values were determined after structure optimization using the same methods and basis sets. FED values were associated with Estradiol carbons as shown by the labeling system depicted in (Appendix-Figure A).

Docking Simulations. Autodock 4.2 was used to simulate the binding between estradiol and HRP. At least ten confirmations were possible for each substrate, and for the purposes of this comparative

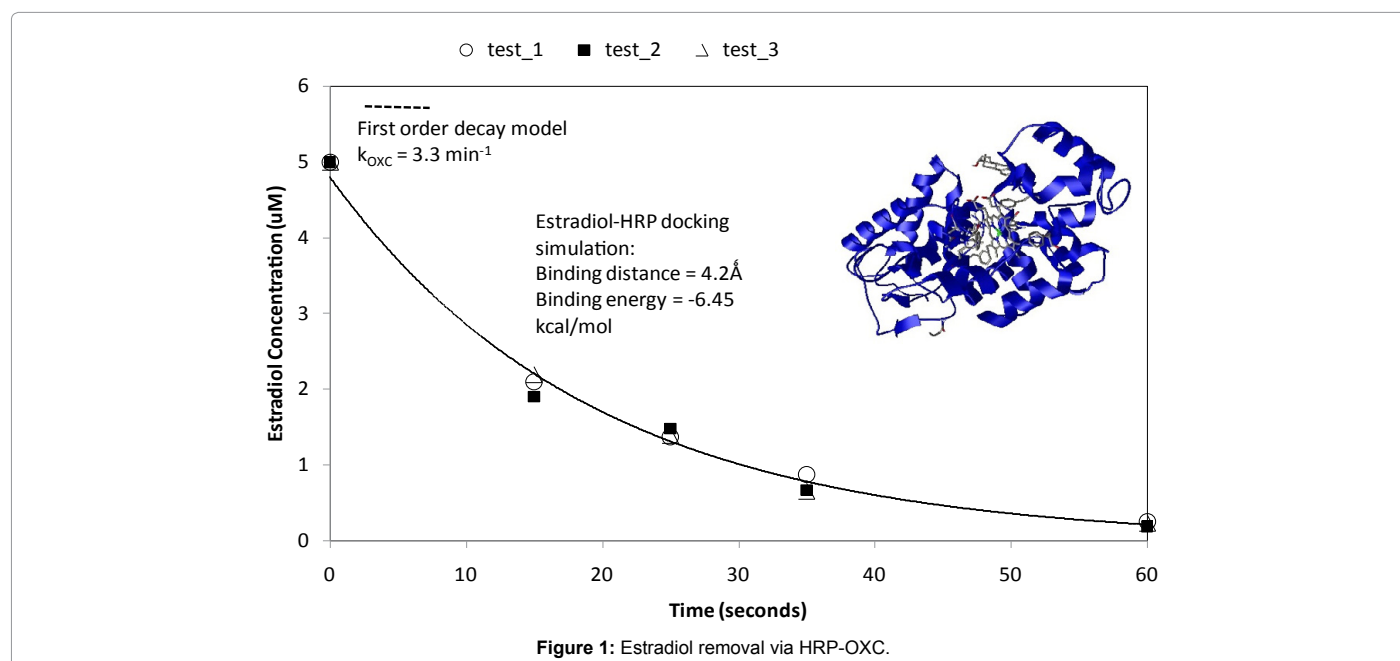
study, the confirmation that was selected had the lowest binding energy because lower energy states are more stable. The Lamarckian genetic algorithm (GA) method was used to calculate free energy changes. In Autodock 4.2, a docking box of 100 × 100 × 100 points was defined with a grid spacing of 0.375 Å. The structural coordinates of the model horseradish peroxidase compound II (1H55) were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSBPDB). Then, the crystallographic water molecules were removed from the active site before docking, and the hydrogen atoms and partial charges were added using the Amber force field. Partial charges were assigned to HRP and the phenolic substrates using the Gasteiger partial equalization of orbital electronegativities method. The coordinates of estradiol were used as the initial position for the docking simulation, and HRP was superimposed onto estradiol to obtain an initial position. The flexible amino acids residues were HIS42, ARG38, PHE41, and ASN70. The binding distance was between the proton associated with estradiol and the imidazole δN on the HIS42 residue as suggested previously [13].

Results

HRP-mediated coupling of E₂

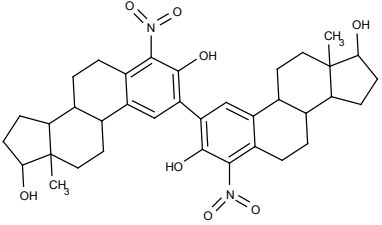
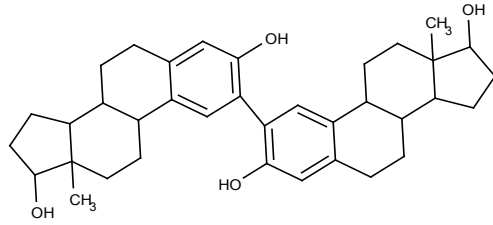
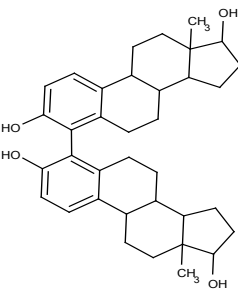
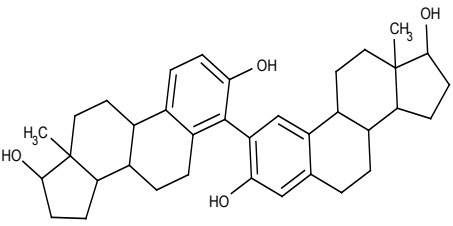
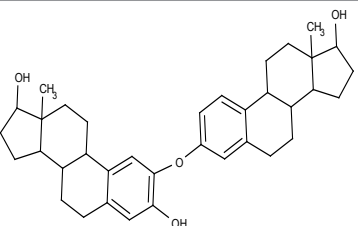
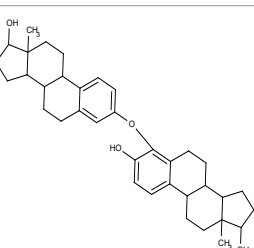
HRP binds very favorably to estradiol, resulting in very fast removal (Figure 1). Data collected in this and other studies [3,4] show that more than 80% of the soluble mass of E₂ is removed from the aqueous phase in under 1 minute. This is much faster than biodegradation reactions, which speaks to the promise of using OXC processes at larger scales. Interestingly, the time scale for precipitate formation is longer, as it takes approximately 30 minutes to visually observe precipitate formation (Appendix-Figure B). Thus it appears that monomers are quickly converted into dimers, but it takes more time to form the insoluble polymers that are eventually separated from water by gravity.

Docking simulations show that the E²-HRP binding distance is 4.2 Å and the binding energy is -6.45 kcal/mol. These values reflect the complementarity of HRP for E₂. The negative binding energy value reflects a favorable interaction because it reduces the free energy of the transition state. The binding distance is relatively short, which also



promotes a fast, favorable coupling reaction. This binding information can be compared to the binding of HRP with a dimer. Since isolating

unique dimers for HRP experiments is difficult, HRP-dimer interactions can be studied using molecular docking. These simulations show that

#	Figure	Formula
1		$C_{36}H_{44}N_2O_8$ (MW=632.31)
2		$C_{36}H_{46}O_4$ (MW=542.34)
3		$C_{36}H_{46}O_4$ (MW=542.34)
4		$C_{36}H_{46}O_4$ (MW=542.34)
5		$C_{36}H_{46}O_4$ (MW=542.34)
6		$C_{36}H_{46}O_4$ (MW=542.34)

Note: Dimer 1 was detected by Penzella et al.[13], Dimers 2-6 were detected by Penzella et al. [14].

Table 1: Dimer structures associated with enzymatically-mediated oxidative coupling of estradiol.

when HRP binds to a dimer, the binding energy and binding distance are -1.12 kcal/mol and 22.7 Å respectively, which is much less favorable than HRP-E2 binding. HRP binds to estradiol much more favorably than with dimers, which suggests that the formation of dimers is more favorable than that of trimers. These bind results are consistent with results shown in Figures 1 and B, which collectively indicate that dimers are formed quickly (<1 min), while the non-soluble higher order polymers are formed more slowly.

Does polymerization occur at the highest fed carbon units?

(Table 1) shows the dimers that have been identified to date. Ring A contains a large fraction of the total FED of E2, and E2-related dimer structures show that these high FED carbon atoms are directly involved in the oxidative coupling reactions. For dimers 1, 2, 5, and 6, oxidative coupling occurs at C3, which has relatively high FED (0.15), and dimer 4 oxidative coupling involves C2, which has one of the highest FED values (0.08). The ether-like analogs shown by dimers 5 and 6 involve carbons C1, C2, and C3 and dimer 3 shows that oxidative coupling involves C1. None of the measured dimer products show oxidative coupling reactions present on rings B, C, or D. These results make it clear that oxidative coupling reactions occur where the FED is relatively high. In principle, this knowledge can be used to model OXC reaction a priori.

It is important to note that OXC depends on a key intermediate, the phenolic radical, which self-couples to produce dimers which are linked by O-C or C-C bonds from the ortho or para (but not meta) position. In the compounds examined in this study, coupling is observed ortho to the phenolic OH because the para-position (10) is blocked by a chemical group. This explains why coupling does not occur at C10 despite it showing the highest FED. Coupling at the other rings is not observed because resonance structures cannot be drawn with a phenolic radical located on those rings. These observations demonstrate that fundamental organic radical chemistry must also be applied when carrying out FED-based analysis.

Electron density shifts

(Figure 2) shows the electron density profile for E₂ and dimer 1.

When E₂ is polymerized, the electron density is dramatically impacted. The FED at C10 is reduced from 0.20 to 0.09, the C3 FED is reduced from 0.15 to 0.08, and the C4 FED is reduced from 0.07 to 0.01.

These results are expected because E₂ is being oxidized. The total E₂ FED is 0.63 while that of dimer 1 is 0.33, which suggests that dimer 1 is not as reactive as E₂. Figure 2 also shows that the FED increases for carbon atoms C5 and C6. This result seems counterintuitive, because oxidation reactions remove an electron from the highest molecular orbital, and would presumably decrease the FED (according to traditional frontier molecular orbital theory). However, some carbon atoms increase in the FED during oxidation reactions. This is redox-induced electron rearrangement (RIER), which happens when nearby electron orbitals reconfigure so that the FED of a particular carbon atom may increase. RIER has been observed computationally in previous efforts [11], but this account is the first in which RIER is shown to happen during the course of an oxidative polymerization.

The electron density shift that is triggered by E2 polymerization is shown in Figure 3 for several measured E2 dimers. These compounds all show significant electron shifting on ring A, which is composed of carbon atoms 1-5 (and 1'-5'). There is also significant electron density shifting for carbon atom 10 (or 10'). Some of the changes are negative because electrons are delocalized, while others are positive due to RIER, and many dimers demonstrate both positive and negative FED shifts. For example when E₂ is converted into dimer 3 the C6 FED decreases by 0.09 while that of C10 increases by 0.09; when dimer 6 is formed the C3 and C10 FED increase by 0.12 and 0.14 respectively while that C2', C3', C5', and C10' FED decrease by 0.12, 0.11, 0.12, and 0.08 respectively. Figure 3 also shows that C10 is a very common point for FED shifting which means that the C10 outer orbital is central to the reshaping electronic profile of E2.

There are two concluding remarks. First, Lund M [12] reported three trimer structures that are coupled at ring A; this observation bodes well for using FED-based theory to predict the location reaction sites for higher order polymers. Future research must detect more trimer byproducts in order to further explore this issue. The final point is that there is both practical and theoretical significance associated with using

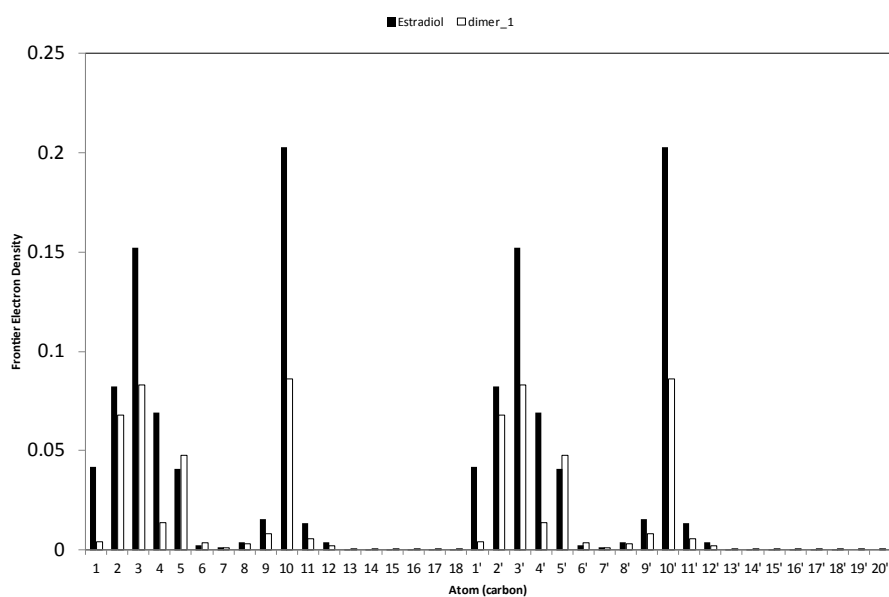


Figure 2: FED Profile for Estradiol and C₃₆H₄₆O₄(dimer).

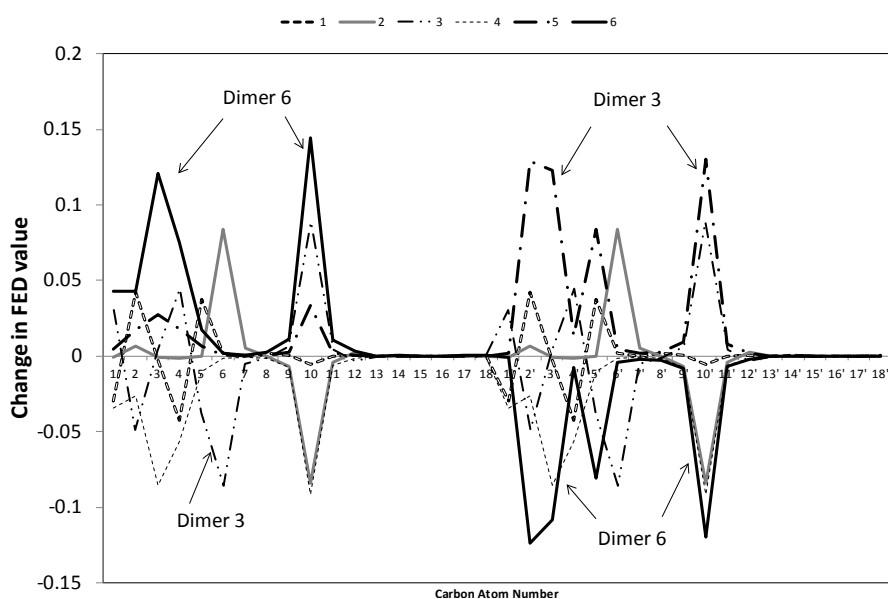


Figure 3: Change in FED values for Estradiol dimers.

FED-based modeling. Practically, the FED-based approach may allow for proactive discovery of the byproducts that emerge from biologically-mediated reactions, including those involving other phenolic pollutants or plant-based lignins, which are known to be subject to enzymatic polymerization at the phenol ring [14]. Theoretically, the FED based approach can reveal insights related to electron transfer dynamics. RIER is an example of such an insight, and the results shown here support the notion that oxidation reactions may allow some carbon atoms to increase in FED.

Conclusions

Oxidative coupling of estradiol monomers proceeds quickly to produce dimer products that do not react as favorably. Experimentally determined dimer structures and FED modeling clearly show that oxidative coupling reactions proceed at carbon units with relatively high electron density. This finding permits the prediction of the identity of polymer products, which can still carry estrogenic effect. These results also show that electron density shifts when dimer products are formed, and that some carbon units gain electron density, because electron orbitals relax and overlap. These findings have a great deal of practical and theoretical significance.

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