Electrochemistry of Iron-Modified Estrogen Binding to the Estrogen Receptor Protein

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Abstract

The focus of this study was on using electrochemistry to probe the energetics of ligand-receptor binding. The estrogen/estrogen receptor system was chosen to look at differences in binding between an agonistic hormone (estrogen) and an antagonistic hormone (a molecule emulating estrogen). An iron-containing estrogen ligand was synthesized, and, to mimic protein binding, was spectroscopically and electrochemically characterized in dimethylformamide (DMF) water solutions using ultraviolet-visible (UV-vis) spectroscopy and cyclic voltammetry (CV). The electrochemistry of the complex in the presence of the estrogen receptor protein indicated that the complex bound to the protein. Future work will focus on surface electrochemistry to analyze the interaction between the ligand and the receptor.

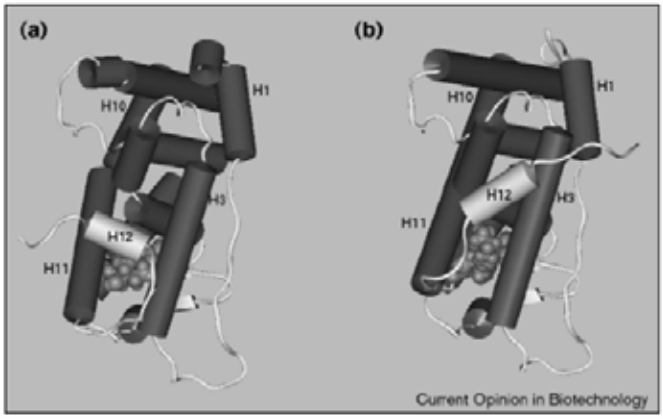

Figure 1. Example of the estrogen receptor protein bound with an agonist (left) and antagonist (right). The lighter alpha helix changes conformation depending on which is bound. Mueller-Fahrnow, A.; Egner, U. *Current Opinion in Biotechnology* 1999, 10, 550–556. (Courtesy of Elsevier Science Ltd. All rights reserved.)

Introduction

Estrogen is a steroid hormone that binds to receptors within cells and, with the assistance of coactivator molecules, causes the transcription and translation of proteins.[1] These proteins are released into the blood stream and cause various biological reactions to occur. For example, estrogen is responsible for female sexual characteristics, the growth of the uterine lining during reproduction, and decreased levels of cholesterol. It may also be linked to regulation of the central nervous system.[2,3]

When estrogen binds, it causes the receptor protein to take on a specific conformation, and this, in turn, starts the cascade of reactions that control the different biological aspects. When an antagonist, or a molecule with a similar structure to the estrogen hormone, binds to the receptor, it changes the protein conformation and prevents these processes from occurring (Figure 1). The purpose of this research is to investigate the energetics of binding of an agonist (a substance that binds to a receptor of a cell and causes a response by the cell) versus an antagonist (a substance that acts against and blocks an action). The conformational change that occurs when an antagonist binds to the receptor is different from the change that occurs when an agonist binds to the receptor, and this difference is expected to be observable.

Estrogen (agonist) ligands with appended iron-cyano (FeCN) complexes have been designed to probe the estrogen-binding event. Antagonistic FeCN ligands will be studied later. FeCN complexes were chosen for their solvatochroism (i.e., sensitivity to the polarity of the solvent). The estrogen receptor protein is known to have a hydrophobic binding site. When the estrogen-FeCN complex binds, it goes from a polar environment (water) to a specific hydrophobic environment in the binding site. It is expected that a significant change will be seen in the UV-vis spectrum as well as in the cyclic voltammetry (CV), because of this difference in polarity. Because antagonistic FeCN complexes will induce a different conformational change in the protein binding site, the FeCN complex will be in a different environment than the estrogen-FeCN complex and should give signature shifts in the UV-vis spectrum and the CV. This experiment advanced understanding of the binding properties of estrogen agonists versus antagonists and may be used in the future to study estrogen binding deficiency in aging women.

Background

The process by which estrogen binds to its receptor protein is relatively clear. The estrogen molecule enters into the cell and binds to its awaiting estrogen receptor. This estrogen-receptor complex then binds to an estrogen response element found on DNA, which causes the binding of its coactivators. Transcription and translation of specific proteins then begin, which eventually cause biological regulation and change.[4] What are not well understood, however, are the events that prevent the active

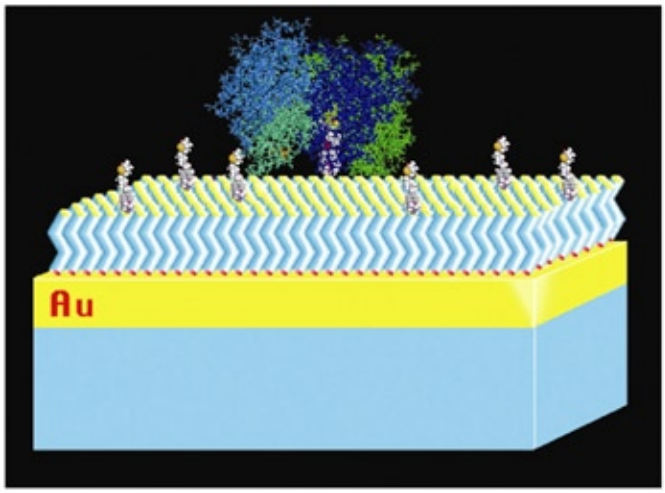

Figure 2. For surface electrochemistry, a monolayer is attached to the gold surface, and the metal-ligand complex is attached to the monolayer.

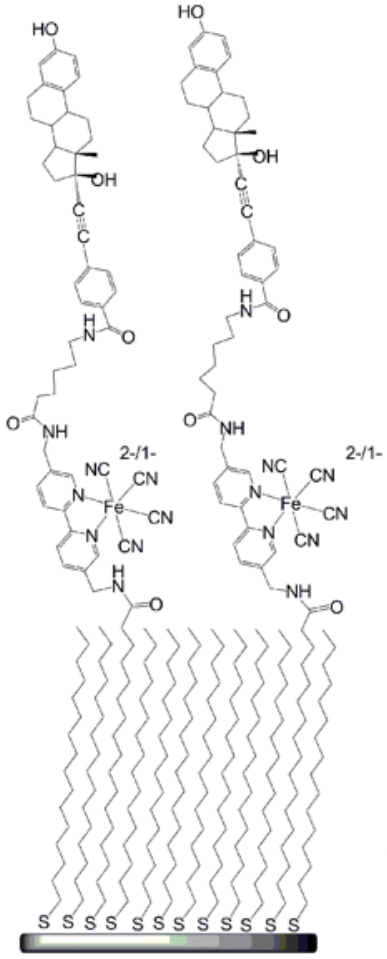

Figure 3. The iron-modified estrogen can be incorporated into the bipyridine monolayer.

binding of estrogen to the receptor protein. This problem is especially important for aging women. The amount of estrogen found in females increases with age until women hit perimenopause, or the stage several years before menopause when estrogen levels begin to drastically decrease as the ovaries produce smaller amounts of estrogen.[5,6]

One of the biggest challenges in hormone research is to understand when a hormone binds to a receptor but causes no change in cell activity. The research of Boger, Jiang, and Goldberg focused on this issue and demonstrated how an antagonist could be changed into an agonist through dimerization, thus improving the understanding of protein receptor activation through ligand dimerization.[7]

Organotransition metal complexes have been used before to label estrogen receptors and test for function. This methodology primarily focuses on the use of ultraviolet light (UV) and the luminescence of tricarbonylrhenium(I) to visualize the binding properties between remodified estradiol and estrogen receptors.[8] In another study, estrogen receptors were labeled with ruthenium-modified estrogen ligands to test for binding. Ruthenium was deemed a superior metal complex to tricarbonylrhenium(I) due to its characteristic high photostability, low-energy absorption, and relatively long-lived luminescence.[9] Both of these studies identified metal complexes that yielded optimal CV results.

In previous work by the Meade group, the biotin ligand was modified with ruthenium pentaammine and iron tetracyano complexes. The electrochemistry of these complexes in the presence of the protein receptor avidin in solution was examined.[10] Mediators were used to show that the protein-bound metal complexes were electronically accessible, but no further information could be obtained. This work demonstrated the possibility of examining ligand-receptor binding through solution chemistry using CV.

Blankman et al found that ω-hydroxyalkanethiols could be used as a monolayer for CV, creating a barrier between solution and electrode, and permitting the direct measure of the reduction-oxidation (redox) couple of a metalloprotein. These energies could then be measured in physiological conditions that mimic the human body, giving a better understanding of how these processes take place in biological systems.[11] When a ligand is bound to a protein receptor, the redox couple shifts because the ligand is shielded from solvent.[12] This knowledge can be used to determine the approximate energy at which a ligand binds to a receptor; a comparison in binding energy can then be made between an agonist and antagonist protein as each binds.

This previous research laid a solid foundation on which to examine the difference in binding energies between agonistic and antagonistic ligands. It provided a start for the synthesis of estrogen ligands containing a metal complex, as well as a comparison for CV data. In identifying the electrochemical difference between agonistic and antagonistic ligands, it may be possible to find more efficient and effective treatments for diseases caused by a deficiency (or surplus) of estrogen in biological systems.

Approach

Electrochemistry, in particular CV, has become an important tool for analysis. Past experiments in the Meade group using CV have focused on the binding of ruthenium- and iron-modified biotin to avidin. Those studies were limited to solution electrochemistry. Current work is focused on the study of how different hormones bind to the estrogen receptor, and whether CV can be used to distinguish between them.

The hormone estrogen was chosen for this experiment for several reasons. It shows a pattern of binding deficiency in aging women, and finding the cause of this malfunction is important for the millions of women affected every year. It might even lead to a decrease in breast cancer mortality, since breast cancer can be caused by an increase in estrogen binding to its receptor protein.[6]

CV is used to determine the redox potential of a metal complex.[13] This technique is known to measure the electron transfer rate between a donor and an acceptor. The electron transfer rate is described by the Marcus Equation.

$$k^0 = \frac{4\pi^2 H_{AB}^{\ 2}}{h\sqrt{4\pi\lambda RT}} \exp[-(\Delta G^\circ + \lambda)^2 / 4\lambda k_B T]$$

where k is the electron transfer rate, H is the electron coupling rate between donor and acceptor, G is Gibb's free energy, and λ represents the energy of reorganization. Two main aspects of this equation are relevant to ligand-receptor binding. First, λ, or the energy of reorganization, is important for surface chemistry (Figure 2). λ is dependent on the polarity of the solvent and should change when the environment surrounding the metal-ligand complex changes. This variable can be determined experimentally using surface electrochemistry techniques. Second, Gibb's Free Energy, ΔG, can be determined in both solution and surface electrochemistry (when the ligand and receptor protein are free to move about and interact in a solution). As the solution environment becomes less polar, the energy needed for the redox reaction to occur lessens, and ΔG should increase.

Two estrogen-FeCN ligands were synthesized by means of methods previously reported (Figure 3).[7,8] One ligand was an estrogen molecule attached to a long-chain carbon (Figure 4). A second ligand was simply an estrogen molecule with a pyridine attached via an alkyne (Figure 5). Using a modified procedure from Coe et al, a pentacyanoiron(II) group was attached to the estrogen ligand to give compound IV, a step essential for electrochemistry.[14] Ligand V was synthesized from the coupling of 4-(aminomethyl)-4'-methyl-2,2'-bipyridine to ligand I (Figure 6). It was then reacted with estrogen to produce ligand VI, which would have been used to make a tetracyanoiron ($FeCN_4$) complex[15,16] if it could have been purified.

To investigate the sensitivity of the estrogen-FeCN complexes to the polarity of the environment, UV-vis spectroscopy and electrochemistry were performed using mixtures of DMF in phosphate buffer, with the concentration of DMF ranging from 0% to 50%. These experiments were designed to mimic protein binding — the polarity of the solution decreases as the percentage of DMF increases. First, the metal-ligand complex was analyzed using UV-vis in the different concentrations of DMF buffer. Cyclic voltammograms of the metal-ligand complex were performed, and the concentration of DMF for each CV experiment was increased using the same percentages as in the UV-vis spectroscopy.

The estrogen receptor protein was isolated from a sheep uterus according to procedures in the literature.[8] The electrochemistry of the estrogen-FeCN complexes was examined in the presence of the protein. Ten µL (1.03 mmol) of compound IV were dissolved in a phosphate buffer solution, and then 200 µL aliquots of a 600 µL receptor protein -5 mL phosphate buffer solution was added. Voltammograms were taken after each aliquot was added.

This electrochemical procedure was carried out only for compound IV. After several attempts, compound III could not be isolated in pure form. The ligand 5-(4-(17α-ethynylestradiolyl)phenyl)-2,2'-bipyridine

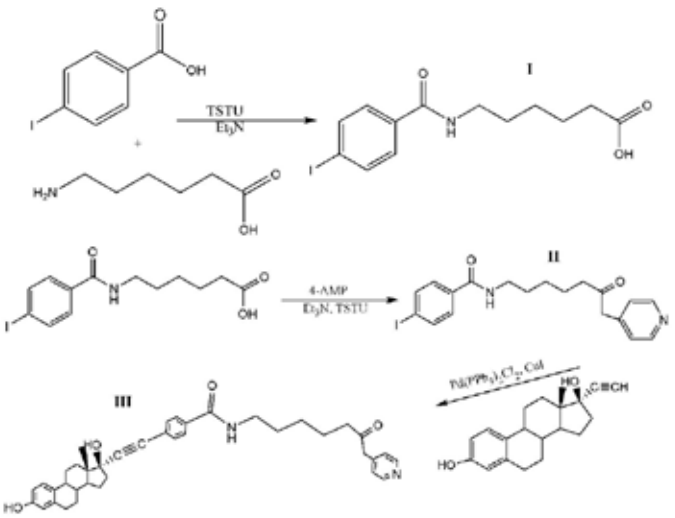

Figure 4. Synthesis of est-C6-py.

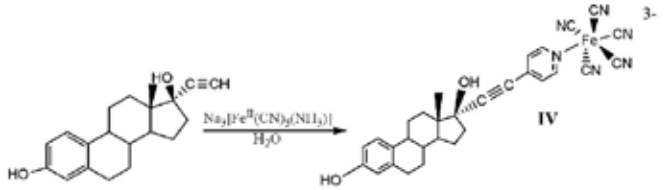

Figure 5. Synthesis of est-py-FeCN5.

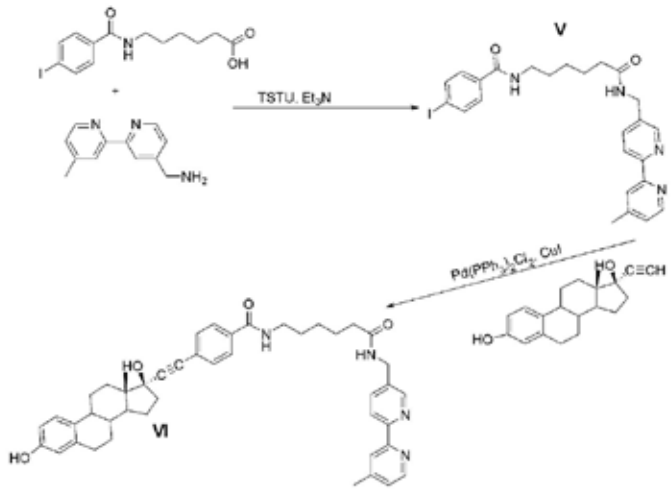

Figure 6. Synthesis of est-C6-bpy.

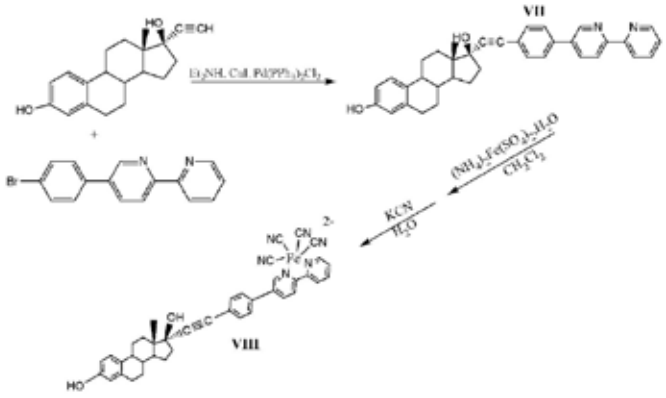

Figure 7. Synthesis of est-phenyl-bpy-$FeCN_4$.

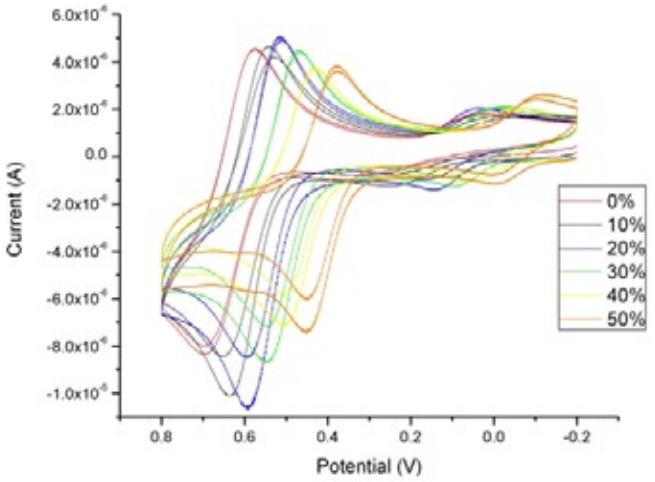

Figure 8. Cyclic voltammogram of est-py-Fe in different concentrations of DMF.

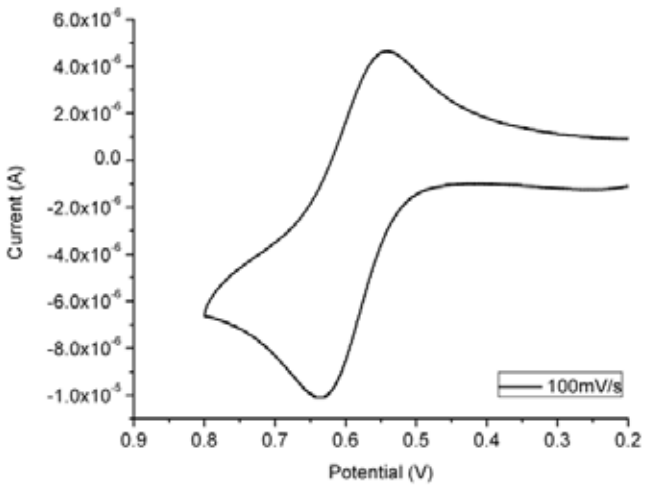

Figure 9. Cyclic voltammogram of est-py-Fe in 10% DMF at 100 mV/s.

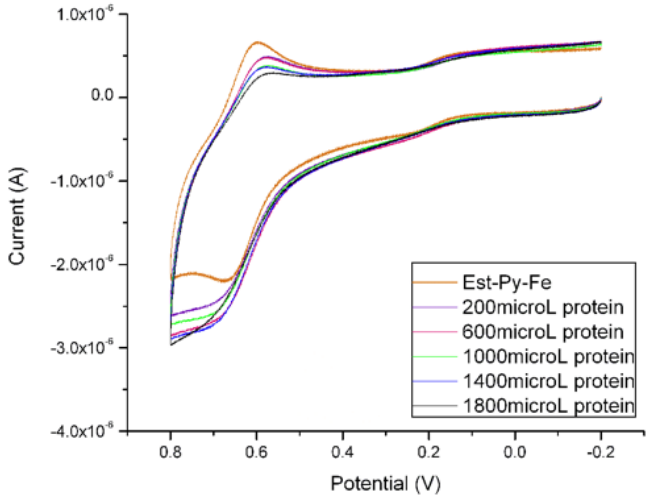

Figure 10. Cyclic voltammogram of est-py-Fe binding to the estrogen receptor protein.

(VII) was synthesized from the reaction of estrogen and 5-(4-bromophenyl)-2,2'-bipyridine (Figure 7).[17] A tetracyanoiron(I) complex was attached to the bipyridine group (VIII), and CV measurement of the molecule was taken.

Results

The ligand-protein interaction for IV was analyzed using solution techniques. First, ligand IV was analyzed using UV-vis in different concentrations of DMF buffer. There were no observable shifts of the absorption band in the UV-vis spectrum after each increased concentration of DMF in buffer, indicating that the metal-to-ligand charge transfer was not as sensitive to solvent polarity as the iron tetracyano complex. CV was performed of the IV ligand in different concentrations of DMF-buffer solution ranging from 10% to 50% DMF. As the percentage of DMF increased, there was a visible shift in the redox couple (Figure 8). This experiment was designed to mimic the hydrophobic environment of the protein-binding site to the metal-ligand complex. The 0% DMF case gives the $E_{1/2}$ of IV (0.585V), or the potential at which the redox reaction between the metal-ligand complex and receptor occurred (Figure 9). This value is comparable to those of similar iron complexes found in the literature.

Ligand IV was dissolved in buffer solution and incremental amounts of the estrogen receptor protein. Cyclic voltammograms were taken after each 200 μL aliquot of estrogen receptor protein solution was added. Although there was no shift in the voltammograms, the peaks did decrease, indicating that the receptor protein was binding to the ligand (Figure 10). UV-vis was not taken of ligand IV with the estrogen receptor protein. Ligand VIII was analyzed using CV, and there were no visible peaks in the voltammogram.

Discussion

Although another research team had already synthesized the modified estrogen ligands that were to be used, some of their published methods did not work on this project. Further, some of the ligands that were

synthesized were not identical to those reported; some reactants and techniques were adapted to the specific needs of the project. These changes caused difficulties in the syntheses of the ligands.

The long-chain ligand II was synthesized and then attached to estrogen to give est-C6-py (III). Compound III was difficult to purify, and after being attached to estrogen, could not be purified using column chromatography. Synthesis of this ligand was attempted twice, but the final yield was insufficient for the next steps of the synthesis to be feasibly carried on. Because this ligand could not be successfully synthesized, it could not be attached to the monolayer, and surface electrochemistry was not an option for analysis.

An estrogen-py-$FeCN_5$ complex was synthesized (IV). This complex was not as sensitive to solvent polarity as previously studied $FeCN_4$ complexes. Electrochemistry of compound IV indicated that protein binding was taking place, although the expected shift in the redox couple was not observed. Binding was indicated by a decrease in current upon addition of the protein, which shows a lower diffusion coefficient of the protein. This result is similar to that found in previous biotin-avidin studies.[10]

Conclusion

An estrogen-py-$FeCN_5$ complex was examined using UV-vis spectroscopy and CV. The $FeCN_5$ complex alone (without estrogen) showed a shift in the CV but not the UV. Addition of protein resulted in a decrease in the electrochemical signal, as has been observed previously for analogous systems. The synthesis of two estrogen-$FeCN_4$ complexes was attempted, but yields were insufficient.

In order to electrochemically study differences between the binding of an agonist and an antagonist to the estrogen receptor protein, future work will focus on using surface electrochemistry. Attempts to synthesize and purify compound VI will continue in order to use surface electrochemistry to provide information about agonist-receptor binding. Also, the ligand synthesis will be adapted so that antagonist-receptor interactions can be studied in a similar way. Surface electrochemistry will be used to measure the interaction between an antagonist (for example, tamoxifen) and the receptor protein so that it can be compared with that of the agonist. If an electrochemical difference can be found between the two, a greater understanding of these binding properties could help engineer future treatments for estrogen deficiency.

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