Structural and Binding Studies of Solvent Reorganization Energy Probes

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Abstract

Electron transfer theory predicts reorganization energy to be one factor that determines the electrochemical potential of a metal ion. This paper describes part of a study that aims to increase understanding of solvent reorganization energy in noncovalent systems by systematically varying the environment around a metal ion, specifically ruthenium. The local environment can be changed in various ways; here a protein is introduced to displace the solvent, creating a very different electronic environment for the metal ion by changing the dielectric constant as well as the refractive index. The *avidin*-biotin system was chosen for study because much is already known about this system, such as the crystal structure. New probes were developed that incorporate biotin and biotin derivatives into ruthenium complexes. The electrochemistry of these complexes was investigated in the presence and absence of *avidin*. A change in the dielectric constant of the surrounding medium is expected to result in a shift in the electrochemical potential of the ruthenium complexes. However, a lack of current signal in the cyclic voltammogram resulted in the presence of *avidin*. Addition of mediators did not resolve the signal, indicating that the protein blocks the metal ion from access to the electrode. Theoretical models developed from calculations indicate that the metal should be openly accessible to mediators, if not the electrode itself. To resolve this discrepancy, structural and binding studies were undertaken. Single-crystal x-ray crystallography was used to further understand the structure of the probes and the protein-ruthenium complex. A crystal structure was determined for one probe, 4-DMP, and its morphology was found to be monoclinic, meaning that two of this crystal's three-unit cell axes of symmetry are perpendicular to one another. The probes were coupled to ruthenium pentaamine and then bound to the protein. These protein complexes were crystallized, yielding a diffraction pattern. Further preliminary kinetic experiments to determine the dissociation rate constant (k_d) were conducted, yielding a value of 1.25×10^{-4} sec^{-1}.

Introduction

The field of electron transfer has a rich history and continues to pose interesting problems for today's researchers. Electron transfer theory predicts reorganization energy to be one factor that determines the electrochemical potential of a metal ion. Studies have been done to investigate other factors that change the rate of electron transfer, specifically the electronic coupling (H_{AB}) and the driving force (ΔG).[1–4] Reorganization energy, λ, is the energy required to change the structure of the reactants, or activate them, from that of equilibrium to that of the products. Reorganization energy can be divided into two parts: an inner sphere component, λ_i, and an outer sphere component, λ_o. The

Marcus Equation

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

Reorganization Energy

$$\lambda = \lambda_i + \lambda_o$$

$$\lambda_o = N_A e^2 \left[\frac{1}{2r_A} + \frac{1}{2r_D} - \frac{1}{d_{AD}}\right]\left(\frac{1}{n^2} - \frac{1}{\varepsilon_s}\right)$$

Peak Potential

$$E_p = E^{0'} + \frac{RT}{\alpha F} \ln\left(\frac{RTk^0}{\alpha F v}\right)$$

Figure 1: Equations for rate of electron transfer, reorganization energy, and peak potential.

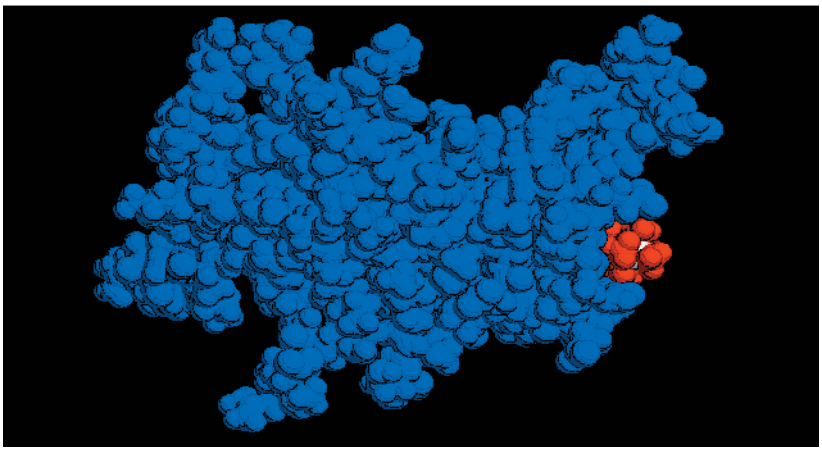

Figure 2: Theoretical model of the *avidin*-ligand complex. The blue represents a single monomer of *avidin,* and red the ligand 4-DMP.

inner sphere component encompasses changing bond lengths and geometries within a molecule, while the outer sphere encompasses intermolecular electronic interactions such as alignment of a polar solvent's dipole moments.

A change in solvent reorganization energy alters the rate of electron transfer between the donor and acceptor. This rate is one factor in determining the peak electrochemical potential of a system (Figure 1). One can easily measure peak potential in standard cyclic voltammetry experiments. This method of analyzing λ through the electrochemistry has never been examined.

Many of the human body's biological processes that are necessary for life involve the binding of small and large molecules by noncovalent interactions. An attempt to study these interactions using electrochemical methods is being made. The *avidin*-biotin system was chosen for study because the binding energy is already known for this complex, and the system can be easily modified. *Avidin* is a tetrameric glyco-protein (with molecular weight of 66 kDa) found in the whites of chicken eggs. Biotin, commonly known as vitamin H, is a small molecule, which is often found in dietary supplements and aids in the metabolism of fats.

The energetics of small molecule–large molecule interactions is related to areas such as drug discovery and signal transduction. Electrochemical experiments have been done by Amanda Eckermann to test the electrochemical potential of biotinylated metal ions with and without the presence of the protein *avidin*. The electrochemical signal should be altered upon addition of *avidin* and return to its original value when the protein is removed because the presence of the protein changes the dielectric atmosphere around the metal. The solvent, water, has a high dielectric constant (ε = 80), while within the protein it is estimated to be much lower (ε ~ 4-20).[5,6] Experiments verified the return of signal strength upon addition of unmetallated biotin, a competing ligand for protein binding. However, loss of a signal upon addition of *avidin* was unexpected and as of yet is unexplained. Theoretical models suggest that the metal complex is near the outside of the protein when bound and not totally buried, as the electrochemical results might suggest (Figure 2). To better understand the local environment of the metal when bound to *avidin*, and to resolve these seemingly contradictory results, research was undertaken to characterize both the probes and the complexes with the protein.

One method chosen to characterize the protein complex is crystallography. This process is twofold: one must grow a single crystal of the substance of interest and then analyze the data collected when passing x-rays through it. A highly focused beam of x-rays impinges on the crystal, and a diffraction pattern is observed. The pattern provides a map of electron density within the crystal, and a mathematical model of the atoms is developed to fit the data. This process is far more complicated for proteins than for small molecules. Analysis of protein crystals must be done using a much higher power and a more finely focused beam of x-rays.

The second method of characterizing the protein-ligand interaction is a binding study. The kinetics of the interaction were studied in an effort to determine the dissociation rate constant, k_d, and the dissociation constant, K_D. Since the rate of combination of the *avidin*-biotin system is already known and available in literature,[7] a comparative study utilizing the high-binding affinity of biotin was the easiest path to access these constants.

Background

Over the past several decades many researchers have investigated the process of electron transfer.[8] "Isotope exchange reactions" were studied as early as the late 1940s.[9] The purpose of much of this research is to accurately predict rates of electron transfer in many types of donor-acceptor pairs. New post–World War II instrumentation also helped further the field by allowing measurements to be taken on a much faster time scale, such as the one needed for fast reactions such as electron transfer. Current electron transfer research is relevant to such fields as molecular devices, artificial photosynthesis, and sensor technology.

Rudolph A. Marcus pioneered much of the modern study of electron transfer. He formulated much of the theory,[8,9] and his equations are considered fundamental. His equation expresses the rate of electron transfer, k_{ET} or k^o, in terms of ΔG (Gibb's free energy), T (temperature), λ (reorganization energy), and H_{AB} (electronic coupling between the donor and acceptor) (Figure 1). Experiments probing H_{AB} have focused on altering the distance and nature of the bridge between the donor and acceptor.[9–11] Further, changing the metal ions changes the free energy, ΔG. However, few experiments have attempted to specifically probe reorganization energy.

An experiment to determine the kinetics of the bonding between *avidin* and the ligand-metal complex is a natural step in this process of understanding small molecule–large molecule interactions. Various kinetics studies have been conducted to investigate the binding of the *avidin*-biotin system.[7,12–14] The binding of *avidin* with small molecules similar to biotin has also been investigated.[12,14] Due to the fact that the binding constant and rate of association of *avidin* with biotin are known ($K_D = 10^{-15}$ M, k = 7 x 10^7 m^{-1} sec^{-1}),[5] a comparison can be made between the ligands used and the original biotin.

Approach

Ligands resembling the small molecule biotin were synthesized using standard organic synthetic methods. Due to the sensitivity of some intermediates to air, the ruthenium complex was synthesized and purified by Eckermann.

The ligand 4-DMP (4-desthiomethylbiotin pyridine) was used to synthesize [(4-DMP)$Ru(NH_3)_5$]Cl_3. This was chosen because of its low λ_i and substitutional stability in both Ru(II) and Ru(III) oxidation states. These compounds have been modified to complex with ruthenium pentaamine via a pyridyl link. We chose biotin and desthiobiotin because their binding with *avidin* is well known and characterized. Their structures and those of all the ligands involved in the greater reorganization energy study can be found in Figure 3. For this study the ligand 4-DMP was synthesized from desthiobiotin and 4-aminomethyl pyridine. It was then crystallized using the overlay method (1 ml of a solution containing 0.01 g 4-DMP in 10 ml of dichloromethane overlaid with 1 ml of hexane). The resulting crystal was analyzed by x-ray diffraction.

Crystallization of the protein was undertaken in collaboration with workers in the Rosenzweig lab (also of Northwestern University). Specific procedures are available describing the crystallization of deglycosylated *avidin*.[10–12] Hanging/sitting drop trays were set up to test many different well solutions and four concentrations of protein, chosen because they are close to the described conditions. This was done in order to find one specific condition most favorable to crystal growth. The

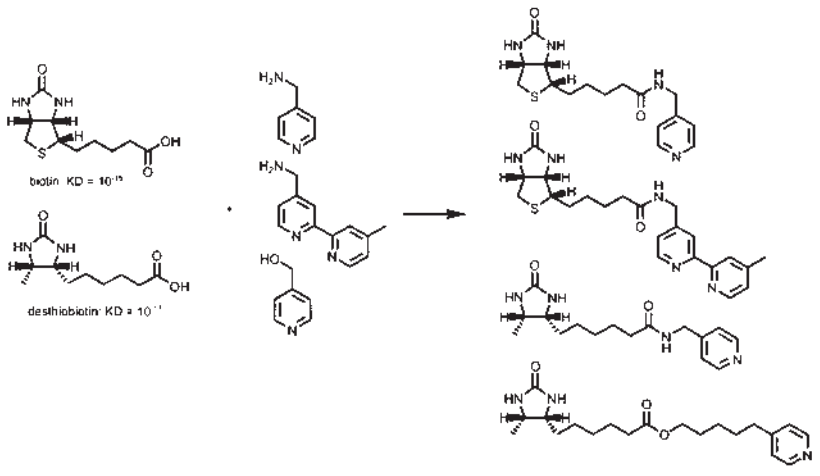

Figure 3: Structures and dissociation constants (with *avidin)* of biotin and desthiobiotin and structures and syntheses of ligands, 4-BMP, bDMB, 4-DMP, and 4-DPEP, respectively. These molecules are all being used to investigate reorganization energy in the Meade research group.

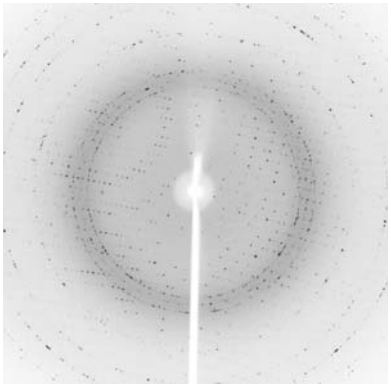

Figure 4: X-ray diffraction pattern of an *avidin* crystal soaked in the ruthenium-ligand complex.

well solutions described in the literature vary buffers in which the protein is stable, such as 0.1 M citrate buffer at pH 5.4, to simple solutions of polyethylene glycol (PEG). Crystals grew under several different conditions in this study, most of which had well solutions 20 percent PEG (20 g of PEG/100 ml of water). A small drop of concentrated (5 mg/ml) metal complex solution was added to drops containing crystals to allow the metal complex to soak into the protein crystals. The soaking process often destroyed the protein crystals by redissolving or disrupting the edges. Those that survived the addition of the metal complex were taken to Argonne National Laboratory and analyzed by Carnie Abajian of the Rosenzweig lab at the Advanced Photon Source. This resulted in the diffraction pattern seen in Figure 4. A magnified photograph of the crystals can be seen in Figure 5.

For the kinetics studies, a solution of the metal complex in water was prepared to a known concentration. Upon addition of an excess of *avidin*, all of the complex binds to the protein. An excess of biotin is then added to this solution. Biotin was assumed to have a higher binding constant than the metal complexes; this is a reasonable assumption because it has the highest binding constant found among small molecule–protein interactions. The metal complexes contain desethiobiotin, known to bind with lower affinity than biotin. After the biotin is added, it replaces any dissociated metal complex within *avidin*'s binding pocket. Small aliquots of the solution were taken at intervals over a period of time. These aliquots were centrifuged using an Amicon® Ultra filter system that allows small molecules, such as the ruthenium ion, to pass through the filter while stopping larger objects such as the protein (30 kDa molecular weight cut off). Thus, the filter will stop any ruthenium bound with the protein, and those molecules not bound will pass through undisturbed. This allows for separating the unbound metal complex once it dissociates from *avidin*. These ruthenium-containing solutions can then be analyzed via inductively coupled plasma mass spectrometry (ICP-MS). This analytical process can detect particles from 1 to 50 ppb with high accuracy. It is sensitive enough to accurately detect individual metal ions where they impact the spectrometer. It counts each ion separately, and thus works extremely well for low amounts of analyte, such as a few parts per billion of metal ion in 5 ml samples. This sensitivity to each impact causes the upper limit of detection to be very low as well, 50 ppb. At higher concentrations there would be less time between impacts of individual particles, causing the machine to count one impact for multiple ions. The spectrometer must be calibrated for each metal; therefore standard solutions known to a very high accuracy must first be run (these solutions are most often bought from commercially available sources). From these ICP-MS data a value of the dissociation rate constant of the ligand-metal complex with *avidin* can be calculated by fitting an exponential decay curve to the plot of the concentration of bound ruthenium versus time (this rate relies only on the concentration of bound ruthenium; thus the data will follow first-order exponential decay behavior).

Results

Crystals of x-ray diffraction quality of 4-DMP were grown using methylene chloride overlaid with ether and reached the size 0.570 x 0.080 x 0.042 mm. Characterization of the ligand 4-DMP was achieved via x-ray diffraction. The cell constants and orientation are consistent with a monoclinic morphology. Data on bond lengths and angles within the molecule and interactions between ligand molecules were gathered (Table 1). Crystals were also grown from the ligand 4-BMP (another ligand in the reorganization energy study); however they were not of the size necessary to analyze.

Crystals of deglycosylated *avidin* were obtained using PEG solutions (Figure 4). Crystals grew in several conditions, as stated above. The general conditions in which crystals grew reproducibly were 15–20 percent PEG in water, and several forms of PEG, from molecular weights of 3000 to 8000. These well solutions were placed in sitting and hanging drop trays. The drops consisted of 1 µl protein solution and 1 µl from the 1 ml well solution. The solutions of protein were 2, 4, 5, or 6 mg/ml in water. The two most favorable conditions were at 20° C, with a protein concentration of 6 mg/ml, and well solutions of 15 percent PEG 3000 in hanging drop trays and 20 percent PEG 6000 in sitting drop trays. Both produced crystals, but sitting drop trays yielded more consistent results.

The crystals of deglycosylated *avidin*, while still immersed in the 2 µl drops, were then soaked in solutions of the ruthenium-ligand complex. This process involves addition of 0.2 µl of a concentrated (5 mg/ml) solution of the metal complex. After this soaking the crystals were analyzed, and the included diffraction pattern was collected (Figure 3). These crystals, however, lacked the size and long-range repetition necessary for solving the crystal structure.

The data collected using ICP-MS to measure the concentration of Ru-*avidin* (the ruthenium-ligand complex bound with *avidin*) as a function of time provided an initial value of the 4-DMP-ruthenium complex and *avidin* dissociation rate constant. These data are consistent with a first-order process. However, this value of 1.25×10^{-4} sec^{-1} is only a preliminary calculation; experiments must be repeated for accuracy.

Discussion

The implications of these crystal studies are very promising. The ligand 4-DMP was successfully crystallized, providing specific structural information such as bond lengths and angles while illustrating the hydrogen bonding that takes place at the ureido ring. This is where most of the binding contacts are in the *avidin*-biotin complex.[15] One may conclude that the modification to include the pyridyl group has not drastically altered the *avidin*-binding properties of the molecule. The diffraction pattern is also encouraging. Growing crystals of a protein that are suitable for x-ray diffraction after the set up of only a few screens of trays bodes well for further efforts. Now that the conditions have been refined, larger crystals can be grown and soaked in solutions of

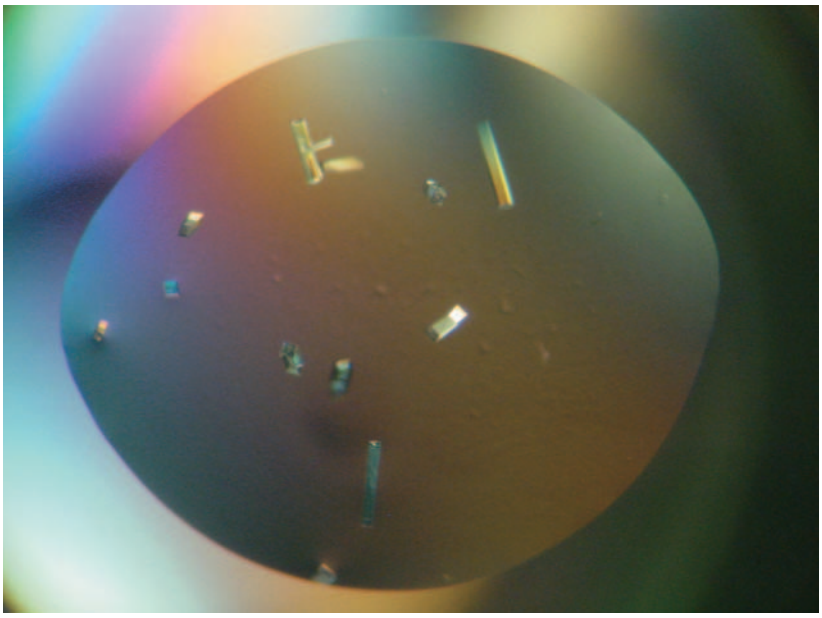

Figure 5: Crystals of deglycosylated *avidin* (size of drop: 2 µl).

Table 1: Crystal data and structure refinement for 4-DMP.

Volume	1649.8(10) Å3
Z, Calculated density	4, 1.226 Mg/m3
Absorption coefficient	0.083 mm-1
F(000)	656
Crystal size	0.570 x 0.080 x 0.042 mm
Theta range for data collection	1.25 to 28.85 º
Limiting indices	-14<=h<=14, -12<=k<=12, -21<=l<=21
Reflections collected / unique	15246 / 7676 [R(int) = 0.0623]
Completeness to theta = 28.85	92.20%
Absorption correction	Integration
Max. and min. transmission	0.9965 and 0.9796
Refinement method	Full-matrix least-squares on F2
Data / restraints / parameters	7676 / 1 / 423
Goodness-of-fit on F^2	0.936
Final R indices [I>2sigma(I)]	R1 = 0.0491, wR2 = 0.1009
R indices (all data)	R1 = 0.0808, wR2 = 0.1120
Absolute structure parameter	-0.8(11)
Largest diff. peak and hole	0.255 and -0.185 e-/Å-3

the ligand, and very accurate data about the nature of their bonding can be collected and duplicated.

The order of magnitude of our preliminary dissociation rate constant results suggests that it is both measurable and that the *avidin*-bound ruthenium complex is stable. The data show that the rate of dissociation is slow enough that characterizing the *avidin*-bound complex should not be impossible, and that the strange electrochemical results are not due to fast kinetics or nonspecific binding.

It is not yet understood why the electrochemical signal is totally lost upon addition of *avidin*. However, much data has been collected now concerning the nature of the binding taking place. As stated above, the unexpected electrochemical result is not a result of unusual kinetics. It is now understood how molecules of the ligand interact, and once the diffraction pattern is analyzed the manner in which the ligand interacts with *avidin* will be fully characterized.

Conclusions

The solid-state structure of 4-DMP is now fully understood. Conditions are known that produce crystals of 4-DMP. Solid-state structures of similar *avidin*-binding ligands would also be beneficial. Now that a few conditions are known to reliably yield crystals of the protein, more trays can be set up that might result in larger crystals. Different soaking techniques can be tried, as well as cocrystallization instead of crystallizing just the protein and then soaking it in the ligand. Crystals of the protein with all the ligands and metal complexes are needed for complete structural characterization of the complex. Conditions and a timescale for the kinetics experiments are now known as well. The k_d results are indicative of a first-order process as expected; however, they are only preliminary and have not yet been repeated. Now that it is known that this technique works and the timescale has been determined, the experiment can be repeated more carefully and accurately.

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