Electrochemical and Binding Studies of Solvent Reorganization Energy Probes

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Abstract

In this study, ligands modified to contain a metal ion were synthesized and their electrochemical properties were examined alone and in the presence of a protein. We chose to use avidin and biotin as a model system. Probes have been developed in which biotin is modified to coordinate to a metal, specifically ruthenium. Binding to avidin displaces solvent molecules and changes the optical and static dielectric constants in the outer sphere environment of the metal ion. The electrochemistry of these complexes was investigated in the presence and absence of avidin. The expected shift in peak potential of the ruthenium complexes was not observed; rather, a lack of current signal in the cyclic voltammogram resulted in the presence of avidin. Electrochemical experiments using mediators were conducted. The mediator, (4-chloropyridine)$Ru(NH_3)_5^{3+}$, and binding complex, (4-DMP)$Ru(NH_3)_5^{3+}$, were synthesized and characterized via UV-visible spectroscopy. Binding between (4-DMP)$Ru(NH_3)_5^{3+}$ and avidin was characterized using HABA assays, which show that the 4-DMP complex binds to avidin in a ratio of four moles of binding complex to one mole of protein, as expected. The dissociation constant for this binding complex was determined to be 1.8×10^{-8} M. Conditions have been determined for the handling of the binding complex and the electrochemical experiments that yield data suitable for simulation fitting. Cyclic voltammetry experiments were conducted at multiple concentrations, and scan rates and simulations are under way to determine the rate of electron transfer.

Introduction

Many vital biological processes in the human body involve the binding of small and large molecules by noncovalent interactions, such as hydrogen bonding and van der Waals forces. For example, binding of substrates to enzymes and the folding of a protein to the tertiary structure are governed by these weak interactions. We are attempting to study these interactions using electrochemical methods. Cyclic voltammetry can be used to determine the rate of electron transfer of species in solution. According to Marcus theory, one term that affects that rate of electron transfer is reorganization energy.[1] The displacement of solvent around a substrate in the presence of a protein alters reorganization energy.

Studies have been done to investigate the factors that change the rate of electron transfer, specifically the electronic coupling (H_{AB}) and the driving force (ΔG) and reorganization energy, λ.[2–5] Reorganization energy 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 inner sphere component encompasses changing bond lengths and geometries within a molecule, while the outer sphere component encompasses intermolecular electronic interactions such as alignment with dipole moments of solvent molecules.

The avidin-biotin system was chosen for study because the binding energy is known for this complex and the system can be easily modified. Avidin is a tetrameric glycoprotein with molecular weight of 66 kDa found in the whites of chicken eggs. Biotin, also known as vitamin H, is a small molecule that is often found in dietary supplements and aids in the metabolism of fats. This protein/small molecule pair bind very tightly, with a dissociation constant on the order of 10^{-15} M.[6] This tight binding is advantageous; we can be sure that the protein is securely bound to the small molecule (binding complex) for the duration of the experiments. Electrochemical experiments are performed on the binding complex alone and, for comparison, in the presence of the protein. Addition of the protein alters the reorganization energy, changing the rate of electron transfer between donor and acceptor (in this case between the mediator and the protein-bound complex). This method of changing reorganization energy by introducing a protein and examining the results through electrochemistry has never been performed.

The energetics of small molecule–large molecule interactions are related to areas such as drug discovery and signal transduction. Electrochemical experiments have been done by Amanda Eckermann to determine the electrochemical potential of biotinylated metal complexes in the presence and absence of the protein avidin.[7] The electrochemical signal should be altered upon addition of avidin. The solvent, water, has a high dielectric constant ($\varepsilon = 80$), while within the protein it is estimated to be much

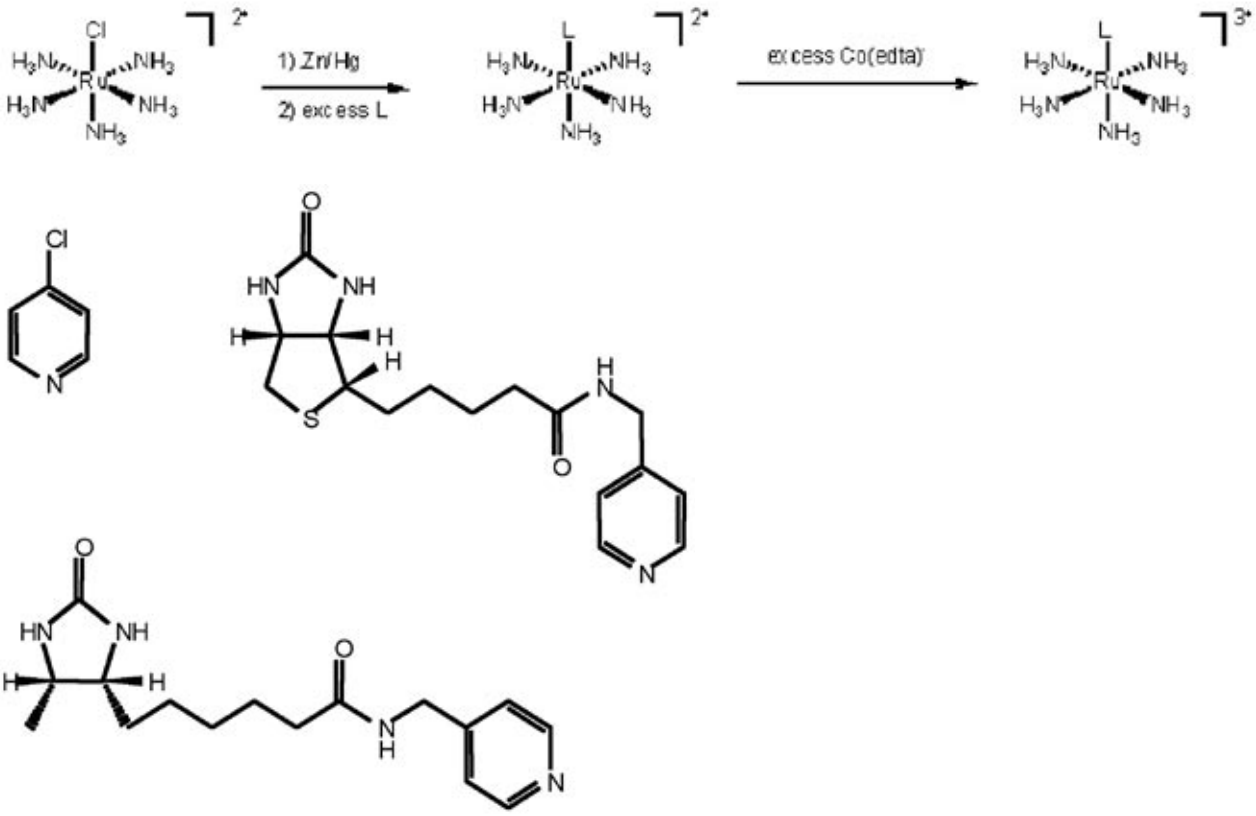

Figure 1: Synthesis of the binding complexes $[(4\text{-BMP})Ru(NH_3)_5]Cl_3$, $[(4\text{-DMP})Ru(NH_3)_5]Cl_3$ and mediator.

Mediator + e- → mediator-	(heterogeneous)
Binding complex + e- → binding complex-	(heterogeneous, slow)
Binding complex + mediator- → binding complex- + mediator	(homogeneous)

Figure 2: Mechanistic equations for simulation fitting.

lower (ε ~ 4-20).[8,9] However, a complete loss of signal upon addition of avidin was observed. Experiments verified the return of signal strength upon addition of unmetallated biotin, a competing ligand for protein binding. 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. Introduction of a mediator, which can shuttle electrons between the bound complex and electrode, should enable communication between them.

Background

Rudolph A. Marcus pioneered the modern field of electron transfer studies; he formulated much of the theory, and his equations are considered fundamental.[5,10] One 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). Experiments probing H_{AB} have focused on altering the distance and nature of the bridge between the donor and acceptor.[10–12] Further, changing the metal ions changes the free energy of the system, ΔG. This study is built upon these theories.

Current electron transfer research is relevant to such fields as molecular devices, artificial photosynthesis, and sensor technology. Many studies have investigated electron transfer in proteins. Blue copper proteins that catalyze important biological reactions are found in many organisms, including humans.[4] It was found that these proteins hold the ligands around the copper in specific positions and this conformation is unaltered upon oxidation or reduction; the proteins also keep water away from the copper's local environment. These two factors significantly lower the redox potential of the metal, which is very important, since the driving force for biological electron transfer reactions is often very low.[4] Other areas of research in this field include electron tunneling through crystals of proteins[13] and the solvent dependence of inner-sphere reorganization energy.[12]

Cyclic voltammetric experiments conducted previously showed that upon addition of the protein, the signal for the binding complex (4-DMP)$Ru(NH_3)_5^{3+}$ disappeared. Electrochemical studies of metalloproteins have shown the electrochemical mediator can often be used to observe the presence of a metal site in a protein.[7] The small molecule, usually a metal complex or an organic salt, has better access to the protein-buried redox site and acts as an electron shuttle between the protein and the electrode.

Approach

A ruthenium pentaammine complex was designed in order to enable communication between the bound complex and the electrode. If the mediator is effective, the current increases above that of the mediator alone when the protein is added. There are very specific requirements for using this simulation software; measurements must be taken at multiple scan rates (the rate at which the electrical potential of the electrode is scanned), and the voltammogram must begin and end at zero. A main goal of this study is to acquire data at multiple scan rates that are suitable for use in simulations. These compounds and their binding with the protein must be characterized.

Both avidin and biotin are available commercially, and this system has been studied extensively. Crystal structures have been reported for a number of species bound to avidin. This system exhibits the strongest noncovalent bonding known, with a dissociation constant of 10^{-15} M.[6] This strong binding is important; it ensures that the binding complex is snugly attached to the protein through noncovalent interactions and not equilibrating in and out of the binding pocket during experiments.

Biotin contains a carboxylic acid at one end, where chemistry can be performed easily without disturbing the rest of the molecule. Most of the hydrogen bonding to residues in the pocket occurs at the ureido ring at the other end of the molecule. It is convenient that a number of known species bind to avidin, allowing us to study variation in the weak interactions.

As previously stated, reorganization energy is made of two parts — an inner sphere and an outer sphere component. The inner sphere component is not related to weak interactions but to bond lengths and angles. An advantage of using octahedral ammine ruthenium complexes for oxidation and reduction is that inner sphere reorganization energy is low for the Ru(II)/Ru(III) transitions. Outer sphere reorganization energy is maximized due to the small, polar ammine ligands.

The mediator, [(4-chloropyridine)Ru$(NH_3)_5$]Cl_3, which was chosen for its potential (71 mV vs Ag/AgCl) and ease of synthesis, and the binding complex [(4-DMP)$Ru(NH_3)_5$]Cl_3 (Figure 1), were synthesized. This binding complex was chosen because of its low λi and substitutional stability in both Ru(II)

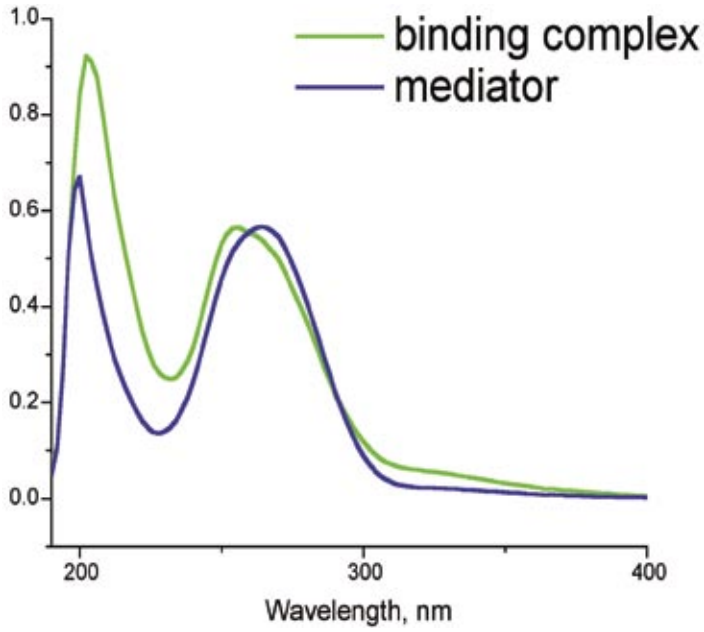

Figure 3: UV-visible spectra of the binding complex and the mediator.

and Ru(III) oxidation states. Ruthenium complexes are generally air stable in the 3+ oxidation state but are less stable as Ru(II). Ruthenium pentaammine pyridyl complexes are easily synthesized according to a method reported by H. Taube.[14] Chloropentaammine ruthenium chloride is reduced using an amalgam of zinc and mercury to form the Ru(II) aquo intermediate, which reacts readily with pyridyl species. These can be oxidized by K_2[Co(edta)] to give the air-stable Ru(III) biotin-functionalized species.[7] The mediator was chosen for its ability, in the 2+ state, to reduce the binding complex. In the mediator the pyridyl ligand is 4-chloropyridine, not a biotin-like compound, in order to prevent binding with the protein and so that it would remain free in solution and able to communicate between the bound complex and the electrode.

The rate of electron transfer can be determined using software that simulates cyclic voltammograms. DigiSim is a software program sold by Biological Analytical Systems (BAS). The input parameters are the mechanistic equations found in Figure 2, the concentrations of both compounds, the diffusion coefficients (which can be estimated or determined experimentally), the potentials of the complexes, the potential scan window, the scan rate, and the area of the electrode that can be determined experimentally. Experiments with the mediator alone can be conducted at various rates, and from these data the software determines the diffusion coefficient and heterogeneous rate for this species. The diffusion coefficient and k_{ET} are also determined for the binding complex alone, as well as for the combination of the binding complex and the protein (the bound species). The simulations of the complexes alone provide information to be used to fit the data of all components combined; from this information, the program can fit simulations to measured voltammograms of all three species combined to determine the homogeneous rate of electron transfer.

The characterization of similar compounds is known from literature.[14] These compounds have absorbances around 250 nm, with extinction coefficients near 5000 $M^{-1}cm^{-1}$. The concentrations of several dilutions of each compound, both the binding complex and the mediator, were measured via ICP-MS and the UV-visible spectra in order to determine the specific extinction coefficient of each compound for further use. Beer's law was employed, using the absorbances measured and the known concentrations from ICP-MS, and the extinction coefficients were calculated.

To characterize the binding between this binding complex and the protein, binding studies were conducted. HABA is a dye that binds to avidin. When bound, it has a characteristic absorbance at 500 nm; this decreases when unbound. The dissociation constant of HABA and avidin is 6×10^{-6} M,[15] significantly larger than that of biotin (10^{-15}) or desthiobiotin (10^{-13}).[6] Therefore, upon addition of the binding complex, which should have a dissociation constant smaller than

that of desthiobiotin but still larger than that of HABA, the dye is replaced in the binding pocket, and the absorbance at 500 nm decreases. Aliquots of both the binding complex and the mediator were added to solutions of HABA and avidin, and their change in the absorbance at 500 nm was recorded.

Further binding studies were undertaken to determine the exact dissociation constant between avidin and the binding complex (BC). This can be measured through dialysis experiments. A solution of avidin and the BC is injected into a cassette. The membrane of the cassette must have a molecular weight cutoff that allows the BC to pass through freely but traps the protein. The cassette is submerged in a buffer solution. The subsequent increase in concentration of ruthenium in the buffer solution is monitored via ICP-MS until equilibrium is reached. Alternatively, the binding complex is added to the surrounding buffer solution, and the decrease in ruthenium concentration is monitored as the complex penetrates the membrane and binds to the protein. Once equilibrium is reached, the cassette is dismantled, and the concentration of ruthenium inside the cassette is measured, as well as the concentration of ruthenium in the buffer. Using the following equation one can calculate the dissociation constant:

$$[B] = [A][Ru]/(KD + [Ru])$$

where B is the bound ruthenium, A represents avidin, and [Ru] is the concentration of free ruthenium.

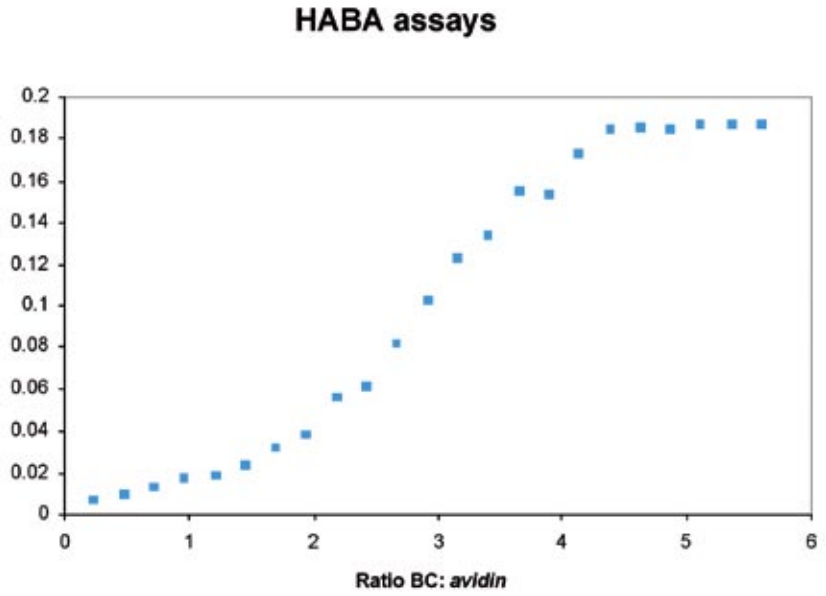

Figure 4: Average of four HABA assays of the binding complex (negative change in absorbance at 500 nm upon addition of binding complex).

Results

The binding complex, $[(4\text{-DMP})Ru(NH_3)_5]Cl_3$, and the mediator, $[(4\text{-chloropyridine})Ru(NH_3)_5$, were both synthesized and characterized by UV-visible spectroscopy and cyclic voltammetry. Measuring the absorbance at five dilutions and the corresponding concentration of ruthenium determined by ICP-MS, the binding complex was found to have a λ_{max} at 254 nm, with an extinction coefficient of 5940 $M^{-1}cm^{-1}$. The mediator was found to have a λ_{max} at 260 nm, with an extinction coefficient of 6300 $M^{-1}cm^{-1}$. The spectra for these complexes in 0.2 M HCl are shown in Figure 3.

HABA assays showed the complex binds to avidin in the molar ratio of 4:1. Assays of the mediator showed no binding, up to five equivalents. The assay of the binding complex can be seen in Figure 4.

Dialysis experiments were conducted to determine the dissociation constant. Cassettes containing both avidin and the binding complex were placed in solutions of potassium phosphate buffer. The increase in concentration of ruthenium in the buffer outside of the cassette was monitored via ICP-MS over time. This constant was found to be 10^{-8} M.

Both the mediator and binding complex are isolated in acidic solution from ion exchange columns. The protein is not

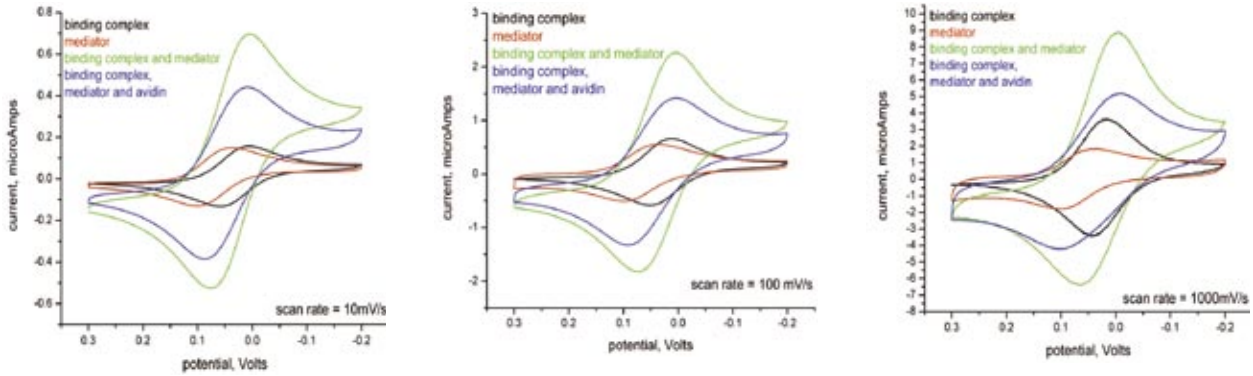

Figure 5: Cyclic voltammograms of synthesized compounds in the presence and absence of avidin at three scan rates. Experiments run to determine the diffusion coefficients and heterogeneous rates of electron transfer for individual species, and then the experiments of all species combined to determine the homogeneous rate, using the DigiSim simulation software.

stable in these acidic conditions. A procedure involving careful evaporation of the acid and precipitation using acetone was established for the isolation of these compounds. Further experiments were conducted in potassium phosphate buffer in which both the compound and the protein are stable.

Electrochemical experiments were conducted on the compounds using cyclic voltammetry. The concentrations of both were varied, and experiments were conducted at multiple scan rates (0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 0.7, 1, 2, 5, 7, 10, 20, 50, 70, 100 and 1000 V/s). The following experiments were conducted: the potassium phosphate buffer background, the binding complex alone in the buffer solution, the mediator alone, those two in solution together, the binding complex with the protein, and finally the binding complex and the mediator and the protein all together. A selection of these experiments can be seen in Figure 5. It was necessary to polish the electrode using 1.0 and 0.05 µm alumina powder, and all of the solutions had to be thoroughly degassed between each experiment.

Discussion

UV-visible spectra were obtained for both compounds and their λ_{max} closely match those of similar compounds published in literature. The extinction coefficients are also in the range expected from literature. The HABA assays confirmed the expected binding of both the mediator and the binding complex to the protein. The dissociation constant of desthiobiotin is two orders of magnitude larger than that of biotin itself. The dissociation constants of compounds similar to this binding complex containing biotin have been published.[16] The similar compounds are Rh-, Re-, and Ir- complexes with biotin-containing ligands, and their constants are in the range of 10^{-10} to 10^{-11}; the constant determined in this study is about two orders of magnitude different from these numbers, thus supporting the reliability of this determination.

One goal of this study was to obtain data suitable for simulation fitting, so as to extract a rate of electron transfer from the electrochemical data. In order to determine the conditions to obtain such data, many conditions were varied. It was found necessary to polish the gold electrode between each experiment. Compounds can adsorb to the outside of the electrode, altering the signal, but this is accounted for by polishing the electrode. Also, solutions required extensive degassing to remove oxygen in solution before experiments could be carried out; oxygen produces a large signal in cyclic voltammetry. It was also necessary to vary concentrations of the compounds in order to observe the mediator activity. If the concentration of mediator was too high compared with the binding complex, no activity could be observed. While cyclic voltammetry is a highly sensitive technique, the concentrations had to be increased for the simulation software. Variation of these conditions resulted in data sets suitable for simulation fitting.

Conclusions

The binding complex [(4-DMP)Ru$(NH_3)_5$]Cl_3 and the mediator [(4-chloropyridine)Ru$(NH_3)_5$]Cl_3 were synthesized and characterized using both UV-visible spectroscopy and cyclic voltammetry. The binding of these compounds with the protein avidin was also studied. Electrochemical experiments were also conducted on this system, and data sets suitable for fitting to simulations were obtained. Simulations to extract the rate of electron transfer are under way.

Synthesis of the corresponding biotin-containing ligand [(4-BMP)Ru$(NH_3)_5$]Cl_3 has begun, but purification methods have yet to be optimized due to complication from the sulfur in biotin. This sulfur can act as a ligand to Ru in the same manner as the pyridyl nitrogen. When the ligand 4-BMP (Figure 1) was allowed to stir for two hours with the Ru(II) aquo complex, the sulfur-bound complex constitutes the product. Overnight stirring resulted in a 50:50 mixture of the nitrogen-bound and sulfur-bound complexes. Optimum conditions for the synthesis and purification of the nitrogen-bound complex must be determined. Once optimized, this compound will be characterized in the same manner as the [(4-DMP)Ru$(NH_3)_5$]Cl_3 binding complex, and the electrochemical data will be collected as for the 4-DMP binding complex. Simulations to obtain rates of electron transfer will be made for comparison.

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