Metalloenzymes and electron transfer proteins influence the electrochemical properties of metal cofactors by controlling the second-sphere environment of the protein active site. Properties that tune this environment include the dielectric constant, templated charge structure, van der Waals interactions, and hydrogen bonds. By systematically varying the binding of a redox-active ligand with a protein, we can evaluate how these noncovalent interactions alter the electronic structure of the bound metal complex. For this study, we employ the well-characterized avidin—biotin conjugate as the protein—ligand system, and have synthesized solvatochromic biotinylated and desthiobiotinylated iron(II) bipyridine tetracyano complexes ([Fe(bpy)(CN)4]2− (1) and [Fe(DMB)(CN)4]2− (2)). The binding affinities of 1 and 2 with avidin are 3.5 × 107 M−1 and 1.5 × 106 M−1, respectively. The redox potentials of 1 and 2 (333 mV and 330 mV) shift to 193 mV and 203 mV vs Ag/AgCl when the complex is bound to avidin and adsorbed to a monolayer-coated gold electrode. Upon binding to avidin, the MLCT1 band red-shifts 20 nm for 1 and 10 nm for 2. Similarly, the MLCT2 band for 1 red-shifts 7 nm and the band for 2 red-shifts 6 nm. For comparison, the electronic properties of 1 and 2 were investigated in organic solvents, and similar shifts in the MLCT bands and redox potentials were observed. An X-ray crystal structure of 1 bound to avidin was obtained, and molecular dynamics simulations were performed to analyze the protein environment of the protein-bound transition metal complexes. Our studies demonstrate that changes in the binding affinity of a ligand-receptor pair influence the outer-sphere coordination of the ligand, which in turn affects the electronic properties of the bound complex.

INTRODUCTION

The properties of coordinatively saturated transition metal complexes can be profoundly influenced by their environment (1−7). The ligands of a transition metal complex can interact with surrounding solvent molecules, macromolecular species, or proteins to affect the electronic state of the metal complex. These noncovalent interactions (known as outer-sphere or second-sphere coordination) include hydrogen bonding, π−π stacking, electrostatic, hydrophobic, and van der Waals interactions (8−11). The concept of outer-sphere coordination was introduced by Werner to explain phenomena such as solvents of crystallization and the formation of adducts between amines and coordinatively saturated complexes (12, 13). In 1981, Stoddart and co-workers presented the first crystallographic evidence of this type of interaction, in which a crown ether is associated with a Pt-ammine complex through noncovalent interactions (14). In recent years, it has been shown that interactions with the second sphere of coordination complexes affect the physical properties. For example, interaction of 18-crown-6-ether with the second sphere of a series of ruthenium-ammine complexes results in changes in the spectroscopic and electrochemical properties of the complexes (6, 7).

Second-sphere coordination is important in biological processes such as iron siderophore recognition, and is currently being investigated as a means to mimic the diverse functionality of metalloenzymes (9, 15, 16). The ability to modulate the properties of transition metal complexes through second-sphere ligand design is of interest for the development of artificial metalloenzymes that combine the features of both heterogeneous and enzyme catalysis (17, 18). Ward and co-workers use chemogenetic protein engineering to optimize artificial metalloenzymes comprising a catalyst anchored to a biological macromolecule (19−21). Second-sphere coordination is known to play an important role in activity and enantioselectivity in these catalytic reactions (17, 19−22).

Previously, we reported a series of redox active transition metal-modified biotin complexes designed to probe the effects of protein binding on the electrochemical response (Figure 1) (23). The biotin—avidin system was chosen as a model due to its strong binding interaction (Kb = 1015 M−1) and extensive structural characterization (24−26). Electrochemical studies demonstrated that the avidin-bound complexes are shielded and unable to effectively interact with the electrode. However, the bound complexes can be accessed via electrochemical mediators, suggesting that the complex is only partially shielded in the binding pocket of the protein (23). Quantitative results could not be obtained for the electrochemical potential of the protein-bound complex. Therefore, to investigate the effects of the protein binding on the electronic properties of the bound complex, we chose to use [Fe(bpy)(CN)4]2− complexes (Figure 1), [Fe(bpy)(CN)4]2− complexes possess electrochemical and optical properties that are highly sensitive to the local environment (4, 27−29). To investigate how differences in ligand-receptor binding energetics influence the second-sphere coordination environment...
of a small molecule when bound to a protein, we designed two redox-active, solvatochromic transition metal complexes that bind to avidin with different affinities. Herein, we directly correlate systematic modifications of the complex environment to observed changes in electrochemical and spectroscopic signals. The influence of avidin binding on the electrochemical and spectroscopic properties of [Fe/(BMB)(CN)]^2^- (1) and [Fe/(DMB)/(CN)]^2^- (2) is discussed in light of X-ray crystallographic, electrochemical, optical, computational, and thermodynamic studies.

EXPERIMENTAL SECTION

Materials and Methods. Deglycosylated avidin was used for crystallization studies and obtained from Belovo, Inc. Egg white avidin, used for thermodynamic studies, was obtained from Molecular Probes. Poly(ethylene glycol) was obtained from Aldrich. K2[Fe(BMB)(CN)] (Fisher Scientific. All other chemicals were obtained from MicroCal, Inc. (Northampton, MA). In a typical experiment, the reference cell was filled with pure distilled water. The reaction cell was filled with 1.8 mL of a 6 µM solution of avidin (24 µM avidin protomer) in pH 7.0 phosphate buffer. The injection syringe was filled with a 300 µM solution of 1. After thermal equilibration to 25 °C, 1 was injected into the reaction cell in 5 µL aliquots every 200 s until saturation was achieved (18 injections). A reference offset of 25% was used. The experiment was repeated injecting 1 into pH 7.0 phosphate buffer. The data were subtracted from the binding data to correct for heats of dilution. The data were fit using the Origin 7.0 OneSites model to determine binding constants (Kb) and heats of binding (∆H). The above procedure was repeated for compound 2.

Crystallization and Data Collection. The K2[Fe(BMB)-(CN)]^2^- complex was incubated with avidin for 1 h at room temperature. The avidin-[Fe/(BMB)/(CN)]^2^- complex was crystallized at 18 °C by using the sitting drop vapor diffusion method. A protein solution containing 3.0 mg/mL avidin bound by [Fe/(BMB)/(CN)]^2^- in phosphate buffer pH 7.0 was combined with an equal volume of precipitant solution comprising 100 mM sodium acetate (pH 4.6) and 25% PEG 3000. The resulting crystals were harvested after six weeks. The crystals were hexagonal, space group P3_121, with unit cell dimensions a = b = 74.20, c = 85.82.

X-ray data were collected at 100 K at the Advanced Photon Source on beamline 5-ID from crystals flash-frozen in liquid nitrogen after soaking in a cryo-protec tant composed of the well solution plus 20% ethylene glycol. The diffraction data were integrated with MOSFLM (31) and scaled by using SCALA (32). The statistics are presented in Table 5.

Structure Determination and Refinement. Phases were determined by molecular replacement using the program PHASER (33) and avidin from the pdb file 1LDO as the search model. The ligand-bound form of avidin was modeled with XtalView (34), refined with CNS (35), and validated with PROCHECK (36).

Molecular Modeling. Force field parameters were derived for [Fe/(BMB)/(CN)]^2^- and [Fe/(BMB)/(CN)]^2^- complexes. Atomic partial charges were taken from the Mulliken population analysis of ab initio geometry minimizations. These calculations were carried out at the Hartree–Fock (HF) level in the program Gamess (37). A Hay–Watt effective core potential was used to describe the iron atoms (38). The carbon and nitrogen atoms in the bipyridyl and cyano ligands were described by 6-31+G* basis set (39). All other atoms were described by the 6-31 G* basis set (40). Geometries and charges were calculated for both 2+ and 3+ metal ionization states.
Iron-ligand bonds were modeled using the points-on-a-sphere methodology (41). In this method, iron–nitrogen and iron–carbon bond lengths are held constant (acquired from crystal structures of similar compounds) (42) and the atom–iron–atom bond angles are determined by the electrostatic interaction of the atoms around the metal center.

The crystal structure of the biotin–avidin complex (obtained from the Protein Data Bank) was used as the starting geometry for the molecular dynamics simulation (26). Hydrogen atoms and other atoms missing from the crystal structure were added referencing standard amino acid residues from AMBER. The ab initio optimized geometry coordinates for the [Fe(bpy)–(CN)4]– complex were appended to the coordinates of the crystal structure of avidin bound biotin for each of the four binding avidin protomers. The energy of the avidin/[Fe(BMB)–(CN)4]2– tetrameric complex was minimized using a combined steepest descent and conjugate gradient minimization technique. The minimized structure was solvated with a 75 Å × 75 Å × 75 Å box of water of approximately 13,800 TIP3P water molecules. The solvated protein structure was minimized using the method described above.

Molecular dynamics (MD) simulations were carried out for the avidin/[Fe(BMB)(CN)4]2– tetrameric complex using the Amber 94 force field (43). Constant volume periodic boundary conditions were employed; the simulation boundary box constructed was slightly larger than the extent of the water buffer used to solvate the protein. A dielectric constant of 4 was assigned to the boundary box to dampen the effects of electrostatics on the system. MD simulations were run for 1 ns recording energy and trajectory information every picosecond. The isopotential surfaces were rendered with HARLEM from a calculation of the electrostatic potential by solving the Poisson–Boltzmann equation using the method of finite differences (44). Electrostatic calculations were carried out using the partial charges on the avidin/[Fe(BMB)–(CN)4]2– complex to represent the charge distribution. The static dielectric constant as a function of position, also used in the Poisson equation, was mapped by defining the low dielectric volume of the protein by a solvent-accessible surface (SAS). A solvent probe radius of 1.4 Å was used to define the SAS. Atoms contained inside the boundary of the surface are assigned a low dielectric constant of 4, outside the surface the dielectric is assigned value of 80 for water. These calculations yield the isopotential surface surrounding the modified ligands. Calculations were made for atoms within a 10 Å shell around the binding ligand. This calculation provides an estimate of the electric field immediately surrounding the ligand and indicates portions of the protein with net positive or net negative charge.

RESULTS

**Electrochemistry.** The $E_{1/2}$ of 1 and 2 in PBS pH 7.2 are 333 mV and 330 mV, respectively. These values are in agreement with previously reported values and comparable to that of [Fe(4,4′-dimethyl-2,2′-bipyridine)(CN)4]2– (312 mV) (23, 45). The $E_{1/2}$ of 1 increases with increasing solvent acceptor number (AN) where AN describes the Lewis acidity of the solvent (Table 1, Figure 2) (30, 46). When DMF is introduced incrementally to an aqueous solution of 1 or 2, the $E_{1/2}$ decreases linearly (Tables 2 and 3, Figure 3). The plot of current (i) vs square root of scan rate ($\sqrt{v}$) is linear for 1 and 2 in all solvent systems. The average $\Delta E_{p}$ (difference of the peak potentials) for 1 and 2 is 64 mV and is consistent for all solvent systems. The $i$/$i_0$ ratio is 0.8 for 1 and 1.0 for 2 in all solvent systems. An electrochemical signal is not observed for 1 or 2 bound to avidin in solution (22). When adsorbed to a mercaptoundecanoic acid (MUA) SAM-coated gold electrode, 1 has an $E_{1/2}$ of 193 mV and 2 has an $E_{1/2}$ of 203 mV. At 100 mV/s, the $E_{1/2}$ for 1 in this experiment is 85 mV with an $i$/$i_0$ ratio of 1.1 (Supporting Information Figure S1). The $\Delta E_{p}$ for 2 is 75 mV with an $i$/$i_0$ ratio of 1.1 (Figure 4).

**Absorption Spectroscopy.** For an aqueous solution of 1, a charge transfer (CT) band is observed at 293 nm and two MLCT bands are observed at 341 nm [metal dπ → bipyridine π* (2 (MLCT2))] and 480 nm (metal dπ → bipyridine π* (1 (MLCT1))) (47). The $\lambda_{max}$ of the MLCT peaks are red-shifted
linearly with decreasing solvent acceptor number (Table 1, Figure 2). When DMF is introduced incrementally to an aqueous solution of \(1\), the \(\lambda_{\text{max}}\) values of the MLCT peaks are red-shifted proportionally (Table 2, Figure 5). Upon addition of avidin to \(1\) in PBS buffer, the \(\lambda_{\text{max}}\) values of the MLCT peaks are shifted back to the original values.

For an aqueous solution of \(2\), a CT band is observed at 293 nm and two MLCT bands are observed at 345 nm (MLCT2) and 486 nm (MLCT1). When DMF is introduced incrementally to an aqueous solution of \(2\), the \(\lambda_{\text{max}}\) values of the MLCT peaks are shifted back to the original values.

Molecular Modeling. In the course of the molecular dynamics simulation, several conformations were found to be accessible to \(1\) when bound to avidin (Supporting Information Figure S2). The conformation in closest agreement with the crystal structure was chosen to compare the second-sphere environment of \(1\) and \(2\) when bound to avidin. In this conformation, the distances from the \(\alpha\) carbon of Ser 41 and the metal atom of \(1\) and \(2\) are 5.76 and 5.92 Å, respectively. The distance between Ser 101 and the metal atom was found to be approximately 10 Å for both \(1\) and \(2\). The distance between Ser 102 and the metal atom was found to be approximately 5 Å for both \(1\) and \(2\). Loss of \(\beta\) architecture in strands 3 and 4 is observed and loops 7,8 and 5,6 are disordered for \(2\) bound to avidin. The models of \(1\) and \(2\) bound to avidin are compared in Figure 7. The isopotential surface of the portion of the protein that surrounds avidin (Figure 6B) and the MLCT2 band shifts 6 nm (495 cm\(^{-1}\)) (Table 3). When an excess of biotin is added to the solution of \(2\)-avidin, the \(\lambda_{\text{max}}\) values of the MLCT peaks are shifted back to the original values.

Figure 3. Electrochemistry of \(K_2[\text{Fe(BMB)(CN)}_4]\) and \(K_2[\text{Fe(DMB)(CN)}_4]\) in increasing concentrations of DMF in PBS buffer at pH 7.2. (A) Representative cyclic voltammograms of \(K_2[\text{Fe(DMB)(CN)}_4]\) (B) Correlation of the \(E_{1/2}\) of \(K_2[\text{Fe(BMB)(CN)}_4]\) with increasing amounts of DMF (C) Correlation of the \(E_{1/2}\) of \(K_2[\text{Fe(DMB)(CN)}_4]\) with increasing amounts of DMF. Cyclic voltammograms were collected at 100 mV/s using a glassy carbon electrode and Ag/AgCl reference. Values are reported vs Ag/AgCl.

Figure 4. Cyclic voltammogram of (A) \(K_2[\text{Fe(DMB)(CN)}_4]\) and (B) \(K_2[\text{Fe(BMB)(CN)}_4]\) adsorbed to a mercaptoundecanoic acid SAM on a gold ball electrode. The cyclic voltammograms were collected at 100 mV/s in 100 mM PBS at pH 7.2 with Ag/AgCl as a reference.

Figure 5. Cyclic voltammogram of (A) \(K_2[\text{Fe(DMB)(CN)}_4]\) and (B) \(K_2[\text{Fe(BMB)(CN)}_4]\) adsorbed to a mercaptoundecanoic acid SAM on a gold ball electrode. The cyclic voltammograms were collected at 100 mV/s in 100 mM PBS at pH 7.2 with Ag/AgCl as a reference.
is mainly positive in nature. This portion of the protein should have favorable interactions with the net negative charge of 1 and 2.

**DISCUSSION**

The redox properties of metal complexes in proteins are influenced by changes in the surrounding protein environment (e.g., cytochrome c and blue copper proteins) (48-50). Metal cofactors bound to proteins experience a solvation environment with a lower local dielectric constant than that of aqueous buffer. The dielectric constant (\(\varepsilon\)) of water is 80, whereas \(\varepsilon\) is estimated to be between 4 and 20 inside a protein (51-53). Changes in the dielectric constant affect the energetics for electronic transitions of metal redox centers. Further, the protein imparts a structured charge environment around the metal complex due to the partial charges of each atom. This charge structure, with its partial positive or negative properties, stabilizes more strongly either the reduced or oxidized state of the metal. Finally, the protein can be viewed as a scaffold containing electrophilic or nucleophilic residues capable of multiple interactions with the metal cofactor.

In order to probe changes in outer-sphere coordination induced by changes in ligand-receptor binding, we have synthesized a biotinylated iron(II) complex (1) and a desthiobiotinylated iron(II) complex (2). These redox-active, solvatochromic transition metal complexes are designed to bind to the protein avidin. Biotin and desthiobiotin bind to avidin with \(K_a\)'s of 1015 M\(^{-1}\) and 1013 M\(^{-1}\), respectively (54). Therefore, weaker ligand-receptor binding is expected to give the \([Fe(DMB)(CN)4]^{2-}\) complex slightly more flexibility within the binding pocket as compared to \([Fe(BMB)(CN)4]^{2-}\). Therefore, the dielectric constant, charge structure, van der Waals, and
hydrogen bonding contacts near the metal will differ for 1 and 2. We have used a variety of techniques to observe differences in the electronic structure of these complexes as imparted by the protein.

**Electronic Structure, Solvatochromism, and Acceptor Number.** Iron(II) tetracyano complexes are known to be highly sensitive to changes in environment (4, 27, 29, 55, 56). \([\text{Fe(bpy)}\text{(CN)}_4]^2-\) is a paramagnetic complex with \(C_2\) symmetry that has completely filled \(\pi^*\) orbitals (\(d_{x^2}, d_{y^2}, d_{z^2}\)), and these orbitals act as the HOMO set for the molecule. Oxidation of the iron(II) to iron(III) species requires removal of an electron from one of these metal-centered orbitals.

The environmental sensitivity of these complexes is largely due to noncovalent interactions with the lone pair electrons on the cyano ligands. Solvent molecules interact noncovalently with these lone pairs. Strong electron acceptors such as water decrease the electron density at the CN and therefore increase \(\pi\) backbonding with the metal ion. This interaction stabilizes the iron(II) state with respect to the iron(III) state. Depending on solvent, the LUMO in \([\text{Fe(bpy)}\text{(CN)}_4]^2-\) might be either the unfilled metal d-orbitals (\(d_{z^2}\) and \(d_{x^2-y^2}\)) or the \(\pi^*\) levels of the bipyridine ligands (28). The first optically allowed transitions from the \(\pi^*\) orbitals, however, are to the \(\pi^*\) orbitals of the bipyridine ligand (47). Weak acceptors such as DMF decrease the \(\pi\) backbonding as compared to water. Therefore, as DMF is added to the system, water molecules are displaced and the iron(II) state becomes destabilized. A solvent with a high acceptor number such as water will result in a higher-energy metal to ligand charge transfer (MLCT) as compared to weak acceptors such as DMF. These changes in MLCT energy are significant enough to be observed spectroscopically. Likewise, stabilization of the iron(II) complex will result in higher oxidation potentials that can be observed by electrochemical methods such as cyclic voltammetry.

The absorbance of 1 in various solvents was measured to investigate the sensitivity of the physical properties of 1 to second-sphere effects. The solvents were chosen to represent a range of ANs. We observed a larger shift of the low-energy MLCT1 band compared to the MLCT2 band. This result indicates that the solvent is interacting with both the cyano and bipyridine ligands to stabilize the \(\pi^*\) orbitals of the bipyridine.
ligand to varying degrees. This phenomenon has been described for cyanoiron(II) complexes by Toma and co-workers and demonstrates that changes in the second-sphere environment around any part of the molecule, whether it is the bipyridine or the cyano ligands, result in spectroscopic changes (56).

As increasing amounts of DMF are added to an aqueous solution of 1 or 2, the energy of MLCT bands decreases linearly (Table 2, Figures 3 and 5). This linear correlation in a binary solvent system demonstrates the effect of an increasingly nonpolar environment and indicates that no preferential solvation of the complexes occurs (56). DMF displaces water molecules from the second sphere of the metal complex, and the complexes do not have a tendency to maintain a solvation shell of water when a nonpolar solvent is introduced.

To investigate the sensitivity of the redox potentials of 1 and 2 to second-sphere coordination, the electrochemical potential \(E_{1/2}\) of 1 and 2 was determined in a variety of solvents. As the AN is decreased, the \(E_{1/2}\) decreases (Table 1, Figure 2). Further, as increasing amounts of DMF are added to an aqueous solution of 1 or 2, the \(E_{1/2}\) decreases linearly (Table 2, Figures 3 and 5).

These solvent experiments provide a basis for rough comparison with avidin-binding results. Upon addition to an aqueous solution of 1 or 2, DMF replaces water in the vicinity of the metal complex; when 1 or 2 binds to avidin, water will be displaced from the metal center in a similar way.

X-ray Crystal Structure and Binding Properties. Isothermal titration calorimetry was used to measure the binding constants of 1 and 2 to avidin. The binding affinities of 1 and 2 for avidin are 3.5 \(\times\) 10^3 M\(^{-1}\) and 1.5 \(\times\) 10^3 M\(^{-1}\), respectively. These values demonstrate a significant decrease in binding affinity compared to that of native \(\alpha\)-biotin \((K = 1 \times 10^{15} \text{ M}^{-1})\) and \(\alpha\)-desthiobiotin \((K = 1 \times 10^{13} \text{ M}^{-1})\). The data fit best to the case where all four binding sites are identical, and binding is independent. Previous studies by Livnah and co-workers demonstrate that when bound to avidin, modification of biotin at the carboxyl terminus by a caproic acid results in a caproic acid results in disruption of the hydrogen bonding of Thr 38, Ala 39, and Thr 40 with the carboxyl terminus of biotin. This disruption results in a decrease in binding affinity (7 to 8 orders of magnitude) for biotin analogues that have an amide at the carboxylic acid (57).

The crystal structure shows two unique protomers (Figure 6). Avidin is a tetramer; the other two protomers are related by crystallographic symmetry. It is apparent that 1 is in a relatively hydrophobic (low dielectric) environment and interacts directly with the protein via electrostatic and hydrogen bonding interactions. While several bidentinated-transition metal complexes have been synthesized, only one other has been structurally characterized bound to avidin (20, 23, 58–61). Similar to the observations of Ward et al., there are short contacts between the metal center of 1 and amino acid residues. Importantly, there is no major reorganization of the host protein.

The three-dimensional structure of 1 bound to avidin shows that the cyano ligands of the iron complex interact with specific residues in the avidin binding pocket. In one protomer, the Ser 41 residue of the L3,4 loop interacts directly with a CN group on the iron(II) complex, and Ser 102 of the L5,6 loop interacts with a CN group through a water molecule. We expect these interactions with the cyano groups to have an observable effect on the electrochemical properties of 1 and 2 bound to avidin. The stabilizing, attractive interaction of the protein with the cyano groups prevents the loops from opening as they do in the cases where the carboxyl terminus is derivatized by caproic acid (40, 57, 62).

In the second protomer, electron densities for the \(\text{[Fe-(bpy)CN]}_{1}\)\(^{2-}\) moiety of 1 and residues 41–44 on the L3,4 loop were not observed. These differences are most likely due to disorder imposed by crystal packing. This conclusion is supported by ITC data that are best fit for a system with four identical binding events.

We have used the program HARLEM to calculate the isopotential surface of avidin and show that the binding pocket of avidin is positive in nature (Figure 8). 1 and 2 are negatively charged and have small polar ligands that should promote interactions with the protein. This portion of the protein should have favorable interactions with the net negative charges of 1 and 2.

Molecular Dynamics Simulations. Molecular dynamics simulations were carried out with 1 and 2 bound to avidin to compare the local environments of the transition metal complexes when bound to avidin. Several similar conformations were found to be accessible to 1 and 2 when bound to avidin in the course of the molecular dynamics simulation (conformations of 1 shown in Supporting Information Figure S2). The conformation in closest agreement with the crystal structure of 1 bound to avidin was chosen to compare the second-sphere environment of 1 and 2 when bound to avidin. For comparison, the distance between the apex of loop 3,4 (Ser 41) and the metal center was measured. This distance is specifically of interest because the crystal structure shows that Ser 41 has a direct interaction with 1. The distance from the \(\alpha\)-carbon of Ser 41 to the iron center in 1 is 5.76 Å, whereas in 2, the distance is 5.92 Å. We did not observe a water molecule between Ser 102 and the cyano group in the molecular dynamics simulation, as was observed in the crystal structure of avidin with 1. The MD simulations show that Ser 101 has a stronger interaction with the binding ligand than does Ser 102. The distance between Ser 101 and the iron center is ~5 Å, while the distance between Ser 102 and the iron atom is ~10 Å. These observations are slightly different than values determined from the crystal structure; however, they are reasonable due to the fluxional nature of the protein in the molecular dynamics simulations. While the location of the ligand within the protein in the crystal structure is very informative, it is only a snapshot of the ensemble of configurations that the metal complex samples during a spectral or electrochemical measurement. The MD simulations further show that, when 2 is bound, the protein structure is more disordered. Figure 7 shows the disorder in strands 3 and 4 and loops 7,8 and 5,6 for 2 as compared to 1 bound to avidin. The environment of 2 is slightly more open and water-accessible than the environment that 1 experiences.

Electronic Affects of Avidin Binding. UV–vis spectroscopy was used to measure roughly the HOMO–LUMO gaps of 1 and 2 while bound to the protein. The optical spectra in aqueous solution show two MLCT bands, one to each of the first two \(\pi^*\) levels of the bipyridine ligands (MLCT1 at lower energies, 480 nm for 1 and 486 nm for 2; and MLCT2 at higher energies, 341 nm for 1 and 345 nm for 2). Upon binding of avidin to 1, the MLCT1 band red-shifts 20 nm and the MLCT2 band red-shifts 7 nm. Upon binding of avidin to 2, the MLCT1 band red-shifts 10 nm and the MLCT2 band red-shifts 6 nm. When an excess of biotin is added to displace 1 or 2 from avidin, the \(\lambda_{\max}\) of the MLCT bands shifts back to the original values reported for the compounds in aqueous solution. These results demonstrate that the outer-sphere coordination of the molecule changes with the ligand receptor binding. The UV–vis studies show that the molecule with the lower binding constant (2) has smaller changes in MLCT bands compared to that of 1, consistent with a higher acceptor number environment.

The spectroscopic shifts upon avidin binding to 1 and 2 are similar to the shifts observed when increasing amounts of DMF are added to aqueous solutions of 1 or 2. The protein binding event displaces water molecules from the metal center just as DMF replaces water molecules that are interacting with the metal center. This solvent system provides a convenient model.
for systematically varying the environment to crudely mimic protein binding.

**Electrochemistry.** The redox potentials of 1 and 2 while bound to avidin were found to shift by −140 and −127 mV, respectively. These potentials were measured by electrostatically adsorbing the protein to a monolayer of mercaptoundecanoic acid (MUA). These dramatic shifts are most likely due to the interaction of the complex with the monolayer as well as the protein. Being sandwiched between the protein and the monolayer minimizes any interaction with bulk water and places the complexes in an even lower dielectric environment compared to avidin in solution. The protein/monolayer combinationdestabilizes the iron(II) state with respect to the iron(III) state, compared with the complex in aqueous solution, raising the energy level of the orbitals that comprise the HOMO. The HOMO is destabilized by 1130 cm\(^{-1}\) and 1050 cm\(^{-1}\) for 1 and 2, respectively.

Immobilization techniques are commonly used to study electroactive proteins (61, 63–70). In our case, a monolayer of MUA was formed and then washed with NH\(_4\)OH\(_{aq}\) in order to leave a number of negatively charged acids on the surface (the pI of avidin is ∼10, and therefore the protein should be positively charged at pH 7). Figure 8 shows the charge distribution at the binding pocket of avidin at pH 7. There is a significant area with a positive charge near the binding site. This positive charge ensures that a portion of the avidin on the monolayer will be oriented so that the bound iron complexes will make effective contact. The observed electrochemical signal is likely from these bound complexes (61). Since avidin is a tetramer, it is likely that a maximum of two of the binding sites are in contact with the monolayer at any given time.

**CONCLUSION**

We have established that the electronic structures of [Fe-(BMB)(CN)\(_4\)]\(^2-\) and [Fe(DMB)(CN)\(_4\)]\(^2-\) are highly sensitive to noncovalent interactions. The optical spectrum shifts up to 20 nm in organic solvents with lower acceptor numbers than water. The shift is linearly dependent on the solvent acceptor number indicating there are specific interactions between the solvent and the CN and bpy ligands. Similarly, the electrochemical response of 1 and 2 in various organic solvents shows a shift that is linearly dependent on solvent AN; shifts of up to 150 mV are observed. These data suggest that the electronic structure of 1 and 2 is sensitive to second-sphere coordination changes and solvation.

The results from solvent studies confirm that the complexes can be used as sensitive probes to investigate the electronic properties of protein binding sites. Binding of 1 and 2 to avidin results in visible changes in the electronic spectra. The shifts are similar to those observed in the solvent studies. We expect the binding event to exclude water from the metal complex, resulting in a lower AN environment. For example, binding of 1 to avidin can be compared to a solution of 1 in 23% DMF in water.

Our data show that the environments of 1 and 2 in avidin are observably different. ITC measurements show that the binding of 2 is weaker than that of 1 by a factor of ∼20. Therefore, we expect 1 and 2 to sample slightly different environments in the binding pocket. Indeed, small differences are observed in both the optical and electrochemical data. Upon binding to avidin, the optical spectrum shifts 20 and 7 nm for 1, while the same transitions shift 10 and 6 nm for 2. The binding of 2 can be compared to 12% DMF in water. The difference in redox potential shifts is small (10 mV), yet well above the error for such a measurement.

The three-dimensional X-ray structure of 1 bound to avidin shows 1 partially buried in a hydrophobic pocket with a direct interaction between the protein and the transition metal complex. An open-loop conformation, expected from the presence of the amide, would prevent the avidin from shielding the complex from bulk water and minimize the observable change in electrochemical potential and absorbance properties. Isopotential calculations, however, show a patch of positive charge near the binding site that should favor binding of the negatively charged 1 and 2. These interactions may partially counteract the effects of having the amide in place of the carboxylic acid. Finally, molecular dynamics simulations show that, when 2 is bound to avidin, the protein structure is slightly more disordered. However, for the MD snapshot most resembling the crystal structure, the distances between the metal center and select residues are nearly the same as when 1 is bound.

Together, these results demonstrate that even small changes in the binding of a ligand (1 or 2) result in small yet experimentally observable changes in second-sphere coordination imposed by the protein. Specifically, these changes include the partial charge structure, van der Waals interactions, and hydrogen bonds. Small changes in these weak interactions are powerful enough to induce changes in the electrochemical and optical properties of the metal cofactors.

The synthetic versatility and the sensitivity of [Fe(bpy)-
(CN)\(_4\)]\(^2-\) complexes to local environmental changes makes these complexes candidates for use in electronic and optical protein detection devices for any number of ligand-receptor pairs. For example, by systematic change of the protein binding ligand (e.g., adding or removing hydrogen bond donors) and observing the effect of protein binding on the spectroscopic properties, the nature of an unknown binding pocket can be evaluated. Further, the shift in the electrochemical potential indicates whether solvent is being excluded from the metal center and therefore can be used to evaluate the hydrophobicity of the binding site.

Sensitivity to small changes in binding affinities indicates that these species can be used as probes for remotely evaluating the noncovalent factors that affect ligand-receptor binding. Our future studies will translate this approach to self-assembled monolayer systems for investigating the effects of protein binding on the electrochemical properties of the metal complex.

**ACKNOWLEDGMENT**

We thank Belovo, Inc. for providing deglycosylated avidin. We thank Harry Gray, Igor Kurnikov, Baudilio Tejerina, and Alec Durrell for helpful discussions. This work was funded by the Nanoscale Science and Engineering Initiative of the National Science Foundation under NSF Award Number EEC-0647560 and Ohmex Corporation. MR thanks the MURI program of the DoD for support.

**Supporting Information Available:** Molecular modeling overlay with crystal structure, isothermal titration calorimetry data, cyclic voltammograms of 1 in 0–50% DMF/water. This material is available free of charge via the Internet at http://pubs.acs.org.

**LITERATURE CITED**


BC900270A