

CHARACTERIZATION AND CATALYTIC APPLICATION OF AN ENGINEERED CUPREDOXIN
POSSESSING A POTENTIAL IN EXCESS OF 1 VOLT

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biochemistry
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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Abstract

An engineered variant of the electron transfer catalyst Azurin - a cupredoxin cloned from *Pseudomonas aeruginosa* - has recently been created, in an attempt to demonstrate the power of the secondary coordination sphere constituents in determining the redox potential of a metal center. This new variant, M44F/N47S/F114N/G116F/M121L (henceforth referred to as 5mAz, for brevity's sake), is an amalgamation of previously characterized secondary coordination sphere mutations known to increase the reduction potential of the active site copper by (primarily) modifying the surrounding hydrogen bonding network. This ensemble of mutations alters bonding interactions and strengths between the C112 and H117 ligands and the copper atom, and in doing so alters both the electron density contributed by the ligand in such a way as to stabilize the reduced, Cu(I) state of the active site. What follows are the results of extensive characterization of this new mutant. While certain aspects of the wild-type protein have not survived such extensive modification (EPR and visual spectroscopy suggests that the active site geometry has been thoroughly altered), the original goal of the construct has been achieved. Years of characterization have yielded data suggesting that this cupredoxin's heavily engineered active site has a reduction potential now well in excess of 1V, opening up a wide range of new possible roles for this protein, including roles not typically ascribed to a mononuclear, type I cupredoxin.

This same cupredoxin, whose reduction potential has been characterized as in excess of 1 volt vs. NHE, appears to promote radical polymerization (in olefins) and oxidative polymerization (in monomers used for the production of conductive polymers). Generation of

polymers based on styrene and pyrrole are discussed herein, as well as characterization of those polymers that have proved promising thus far. The protein used appears to have several advantages over a conventional inorganic or organometallic polymerization catalyst, including: activity in the presence of oxygen, activity in water, and activity at room temperature, as well as potentially tactic polymerization capability. These advantages may make a protein-based catalyst of interest when aqueous functionality is necessitated. Moreover, other possible catalytic chemistries of interest are described that have only been discovered in recent weeks, and while not thoroughly characterized, do suggest possibly fascinating new functionalities and redox states imparted by the effects of the highly altered reduction potential and geometry of the active site of this protein.

A separate project involving the production of a laccase protein (*Streptomyces coelicolor*) was undertaken, beginning from a synthetic gene. A protocol for the growth, harvest, and purification of this protein was developed, though optimization of the process is still needed. As laccases are potent oxidizers of organic substrates, show the potential to be used as fuel cell anodes, and contain all 3 common types of cupredoxin active site (type I, II, and III), the effective large-scale production of a modifiable laccase would be useful. This particular laccase is both small (allowing it to have high electrode densities), and multimeric (forming a ring of 3 monomers), with 3 active sites per trimer generated (active sites present at each interface of the individual monomers). Modifications to the interfacial residues and their hydrogen bonding network, hydrophobic interface, and electron transfer network, may prove useful in the design of both binding sites for specific substrates and in improving electron transfer rate for use in fuel cells, both by optimization of the electron transfer route and the reduction potential of the gateway type I copper site.

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CHAPTER 1: CHARACTERIZATION OF M44F/N47S/F114N/G116F/M121L AZURIN – AN ELECTRON TRANSFER CATALYST WITH AN ENGINEERED APPARENT OXIDATION POTENTIAL IN EXCESS OF 1 VOLT

Introduction

Redox proteins are a vital component of the proteome of every organism in nature, and catalyze the most difficult reactions necessary for life¹. The enormous diversity of environments in which organisms have adapted and thrived has resulted in a wide array of redox protein active sites, structures, and functionalities¹. In turn, our study of these enzymes allows us to understand the complex and at times previously unknown chemistries that nature uses to accomplish a variety of tasks. Due to their close involvement with the metabolic processes of all organisms, redox proteins are important targets for both understanding and treating disease, as well as in understanding how organisms have adapted to survive in harsh environments and manage to live on exotic sources of energy. In achieving a greater understanding of the mechanisms by which organisms utilize their environment for their survival, we can utilize them for our alternate purposes.

Electron transfer events lie at the heart of all metabolic processes, and man-made catalysts are often dependent on electron transfer events for their core function. Type I cupredoxins are found throughout nature, and combine both exceptional electron transfer efficiency and a broad range of naturally occurring reduction potentials (~100-1000mV). This has led to their incorporation in a number of processes – including respiration and photosynthesis.^{2,3,4} Their exceptional properties also make them an important subject of study for chemists, as nature has made a truly remarkable accomplishment in turning copper – an

ordinarily poor electron transfer agent, limited in its capabilities by its primary ligand set and solvent environment⁵ – into an extraordinary outer *and* inner sphere redox catalyst. Reverse engineering of this functionality is therefore of significant interest to inorganic chemists, and the small cupredoxin, Azurin, provides an ideal test-bed for probing the effects of changes to the secondary coordination sphere.

While alterations to the secondary coordination sphere are difficult in purely inorganic systems that function in organic solvents, protein based systems provide a unique means by which to tweak the functionality of an active site with very remote changes to the structure of the protein⁵⁻⁷. Hydrogen bonding networks and solvent isolation in Azurin allow for significant changes to both electron transfer rate and potential, allowing the wild type, with a relatively mild reduction potential of 350 mV vs. NHE, to be turned into a comparatively potent biological oxidizer. At the outset of this project, a goal of 1 V vs. NHE was set, as near this potential a number of interesting chemistries become possible, including water oxidation⁵. In the process of altering ligand-metal interactions via secondary coordination sphere alterations, the spectroscopic features of the copper binding site diverged significantly from those of the wild type protein. This divergence indicates geometric distortion of the site and likely impaired electron transfer efficiency. The altered spectroscopic features have been tentatively ascribed to lowered stability of ligand binding of one of the primary histidine ligands (H117), allowing for an equilibrium between bound and un-bound to be established. In literature, it is noted that the H117G mutation creates an open ligand binding site⁸ – as a consequence, we set out to show evidence that this may be the case for the 1 V Azurin system. As well, due to its poor electron transfer characteristics^{8,9}, the 5mAz variant proved to be particularly difficult to characterize, likely due to poor interactions with cyclic voltammetry electrodes and techniques used in the past. Therefore, in order to take advantage of the unique properties of a cupredoxin possessing

a reduction potential exceeding 1 V, we set out to prove that this system indeed had such a potential, and that it suffered from the problem of ligand instability. In doing so, we have found that – while still flawed as an outer sphere electron transfer catalyst – the 5mAz variant has several unique properties that make it of substantial interest.

Materials and Methods

Preparation of *P. aeruginosa* Azurin

Azurin variant M44F/N47S/F114N/G116F/M121L was prepared via expression in *E. coli*. Cells were transformed with the Azurin-containing pET9a vector with the appropriate mutations to the gene sequence and were selected for using Kanamycin when plated. Overexpression was accomplished in BL21DE3 STAR cells from Invitrogen. Overnight cultures of the BL21DE3* transformation were prepared by inoculation of 5 mL LB (Lysogeny Broth) incubated at 37C, containing 100 ug/mL Kanamycin. After overnight incubation, 5 mL LB culture was added to 2L 2xYT (32g Bacto-Tryptone, 20g Yeast Extract, 10g NaCl) broth in 6L flasks. Cultures were grown at 37C, shaking at 210 RPM, until an OD₆₀₀ of 1.5 or more was reached, followed by a period of 4-5 hours of induction using approximately 200mg/L IPTG at 37C. After the induction period, cells were harvested by centrifugation in 450 mL centrifuge bottles at 8000 RPM (ca. 10,000xg) for 10 minutes. Cells were then resuspended in their centrifuge bottles using a total of 1L sucrose solution (20% sucrose, 0.5g EDTA, 5g TRIS-Base, adjusted to pH 8 with HCl; 150 mL per bottle), and shaken in the solution for 1 hour at room temperature. Cells were re-pelleted at 10,000xg for 10 minutes, and 1L osmotic shock solution (0.98g NaCl, 4 mM DTT, chilled to 4C) was added to the pellets to rupture the periplasmic membrane. Cells were shaken with the osmotic shock solution at 4C for 10 minutes, and were subsequently re-pelleted (10 minutes, 10,000xg). The supernatant was collected, and contaminants precipitated from the supernatant using a volume of precipitation solution (400mM NaOAc, pH 4.0) equivalent to 10% of the

osmotic shock supernatant. After precipitation, cells were once again centrifuged (10 minutes, 10,000xg). The resulting supernatant was collected, and incubated with SP-sepharose beads (GE Healthcare) equilibrated with pH 4.1 Ammonium acetate for approximately 2 hours under gentle shaking. The beads were then added to an FPLC column, and a customized gradient was run on an Akta FPLC (GE Healthcare) to remove further impurities. The gradient was composed of a 2-buffer system: 50 mM Ammonium Acetate – Buffer A: pH 4.1; Buffer B: pH 6.35. A step gradient was performed with 3 steps – 55%B step to remove impurities, and a subsequent 100%B step to elute the partially purified Azurin, which was then kept for further purification. The eluted Azurin was then concentrated and further purified using a Q sepharose column (method: column equilibrated with 100% buffer B, causing protein to run through while contaminants were retained on the column). In the final purification step, a size exclusion column (S-100 beads, GE healthcare) was used to separate different variants of the mutant as well as remaining contaminant protein.

Azurin Titration with Cu²⁺

Azurin was titrated with Cu²⁺ or Cu¹⁺ according to experimental need. Copper was added slowly and in 1/10th equivalents (calculated using $\epsilon_{280} = 9800\text{mM}^{-1}$), on ice and with rapid stirring in a glass test tube, and with 3-5 minutes between additions. The absorbance at 420 nm and 600 nm was monitored between each addition, with the ratio between the 600 nm Cu-S MLCT band and Tryptophan 280 nm absorbance monitored in order to determine when saturation binding has been reached (R/Z between 5 and 10 depending on quality of the protein).

Characterization of External Ligand Binding

A 250 μ M solution of 5mAz titrated with Cu^{2+} was incrementally exposed to external ligands (Cl^- , Br^- , and Imidazole). Chloride ion had no discernible effect as compared to simply increasing ionic strength. Bromide ion was added in aliquots from a 6M solution of KBr (pH 6.5 KPi) that increased Br^- concentration in 10 mM units up to 100 mM, and from there in 50 mM units up to 500 mM. Imidazole was added in aliquots from a 2M solution of Imidazole HCl titrated to pH 7.2 using HCl and NaOH, on a gradient increasing imidazole concentration from 0-100mM in 10 mM steps.

EPR of 5mAz

5mAz was prepared at a range of pH values using UB buffer (Universal Buffer: 50 mM NaOAc, 40 mM each MES, MOPS, TRIS, CAPS), and using KPi (50 mM) to obtain the scan at pH 12. Protein concentrations were generally approximately 0.5 mM, with 20% v/v glycerol added as a glassing agent. EPR spectra were collected using an X-band Varian E-122 spectrometer at the Illinois EPR Research Center (IERC).

Cyclic Voltammetry of 5mAz

Cyclic voltammetry of 5mAz was performed differently from all other Azurin variants, due to difficulties in reproducibly resolving the reductive peak. A new method was developed, in which graphene oxide (graciously prepared and provided by Longhua Tang) was deposited on a pyrolytic graphite edge electrode from a saturated aqueous solution of the material. After adding approximately 20 μ L of the saturated solution to a 1 mm diameter round electrode surface, and allowing the material to dry, protein solution (10 μ L of a 10-100 μ M, pH experiment dependent) was added, and a 1 cm^2 piece of dialysis tubing (pre-wetted, washed to remove any latent N_3^- , 12 kDa molecular weight cutoff) was placed over the droplet and secured using a circular rubber gasket. As a consequence, an extremely thin layer of highly concentrated

protein solution was held in close contact with the electrode surface. The electrode was then scanned through a range of potentials and rates, dependent on what was called for by the experiment, but typically between -0.6V and 1.5V vs. Ag^+/AgCl . All scans were conducted under Argon, and in an electrolyte solution sparged with Argon for at least 15 minutes prior to use.

Results

UV-visible spectroscopy of the 5mAz protein and subsequent titrations using external ligands (Br^- and Imidazole) indicate that H117 may indeed un-bind from the active site of the protein. Literature absorbances for the H117G variant of wild-type Azurin match almost perfectly with those found in the 5mAz variant. **Figures 1 and 2** show substantial, non-linear shifts of both the intensity and position of the 420 nm and 600 nm bands as bromide ion concentration is increased. The use of a histidine-mimicking ligand, imidazole, has an even more dramatic effect on the spectroscopy of the protein, causing a multi-step shift in both position and intensity of both bands with increasing concentration (**Figure 3**). Interestingly, only the 420 nm band tends to shift overtly with increasing Br^- , with a second, 550 nm peak appearing at much higher concentrations. However, imidazole titration blue shifts the 600 nm peak, and red shifts the 420 nm peak, while simultaneously causing shifts in intensity over a relatively small range of concentrations. Interestingly, while literature indicates that imidazole completely recovers the H117G 600 nm peak's wild-type intensity and position, no such pattern is noted here – the final product is a reddish yellow color.

EPR of 5mAz at a variety of pH values (**Figure 4**) shows a heavy dependence on pH, and further may indicate the presence of hydroxide bound at the active site above pH 8. Lower pH values (between 4 and 6) seem to show at least some similarity to the wild type, while higher values are indicative of multiple unique species.

Despite a warped active site structure, CV was successfully obtained using the new technique outlined in the materials and methods. The use of a Cu(II) titrated 5mAz sample, prior to size-exclusion column purification, showed both an oxidative and reductive peak at 1000 and 797 mV vs. NHE respectively (**Figures 5 and 6**). With higher purity material (size exclusion column purified 5mAz Azurin), and Cu(I) titrated in an attempt to prevent damage from self-oxidation (seen as many anomalous peaks, **Figure 7**), a tremendously high reduction potential was found of 1.247V vs. NHE (**Figure 9**). Several unusual peaks at highly reducing potentials were also noted, and as there are fewer than 3 reducible residues in the protein, this may indicate the presence of some unusual oxidation state or a modification to the protein (**Figure 9**). As well, in the process of redox property characterization, some indications of a potential catalytic activity were noted, which appear to overlap with the same potential region in which water and ammonia oxidation occur^{10,11,12} (**Figure 10**).

Discussion

Variants of Azurin possessing mutations to the M121 axial methionine ligand have been noted previously to possess unusual visible-range spectroscopic characteristics (usually, a “red” peak between 400 and 500 nm). The Cu(II) state of 5mAz was noted to have the most significant red peak while still retaining its 600 nm Cu-S metal to ligand charge transfer band. As mentioned previously, literature indicated that this absorbance may arise from binding and unbinding of Histidine 117 to the copper at the active site. However, having performed further purification using size exclusion, we have found that there may be multiple species (perhaps multiple folds) of Azurin that contribute to form this final spectrum, with one form contributing primarily the red 420 nm peak (similar to a type II copper site)^{6,13}, and the other primarily contributing a 600 nm band as would be expected from a type I copper site.

Interestingly, these higher purity size exclusion column products, combined with a variation on normal electrode construction, allowed us to identify a very high purity, extremely high potential product, with an evident potential of 1.247V vs. NHE. This product could only be isolated when working with protein titrated with Cu(I) that had never been previously oxidized. The lifespan of Cu(II) titrated 5mAz Azurin appears to be significantly limited, as is apparent from the number of redox active species generated from a relatively young sample (**Figure 7**). Oddly, even in the stable Cu(I) form, a number of redox-active moieties are noted at very high reductive potentials, which could not be easily attributed to any one redox active residue (**Figure 9**), and may represent some chemically interesting intermediates created by a protein with such a high reduction potential.

Due to its similarity to the H117G mutation created and characterized by Jeuken et al., we examined the external ligand-binding properties of the mutant, and found that a variety of electron-donating ligands appeared to interact with the copper site (**Figures 1-3**). This unusual property, while not beneficial for effective long-range electron transfer, may prove to be useful in the future for the design of oxidation catalysts using the protein, as the open site reveals the possibility for inner sphere electron transfer reactions. Elimination of the H117 residue, which no longer appears to effectively bind the copper site, and its replacement with another residue that both promotes copper retention and allows substrate binding will prove useful for the design of a catalytic cupredoxin in this protein scaffold. As well, the introduction of external ligands or small molecules that cross link to a residue at position 117 and also bind copper may restore some long-range electron transfer capability, and indeed impart novel properties on the active site of the protein.

The potential uses of such a high potential Azurin variant are currently limited by the self-destructive nature of a protein with such a high potential – any oxidizable residue accessible

by the active site (i.e. Tryptophan, Tyrosine, Cysteine, etc.) presents a potential oxidation target. Future variants may in fact have to focus on reducing the potential of the protein in favor of generating a stable cupredoxin, and will as well have to focus on preventing warping of the active site, and perhaps even going to far as to remove all oxidizable residues. Perhaps just as importantly, a more stable variant of this protein may be far more effective at taking advantage of the unknown catalytic properties it has been recently found to have. A very large oxidizing current catalytic wave is noted at potentials above 900 mV (vs. Ag^+/AgCl). This wave appears to be slightly dependent on the presence of oxygen and on the buffers present, and resides in the region where water oxidation catalytic features are noted. Due to the unusual extra redox active species noted at highly reducing potentials, as well as an open binding site and a solvent-exposed histidine that could potentially be used to bridge two binding sites and two or more copper atoms, it seems possible that ammonia or water oxidation may be occurring.

Chapter 1: Figures

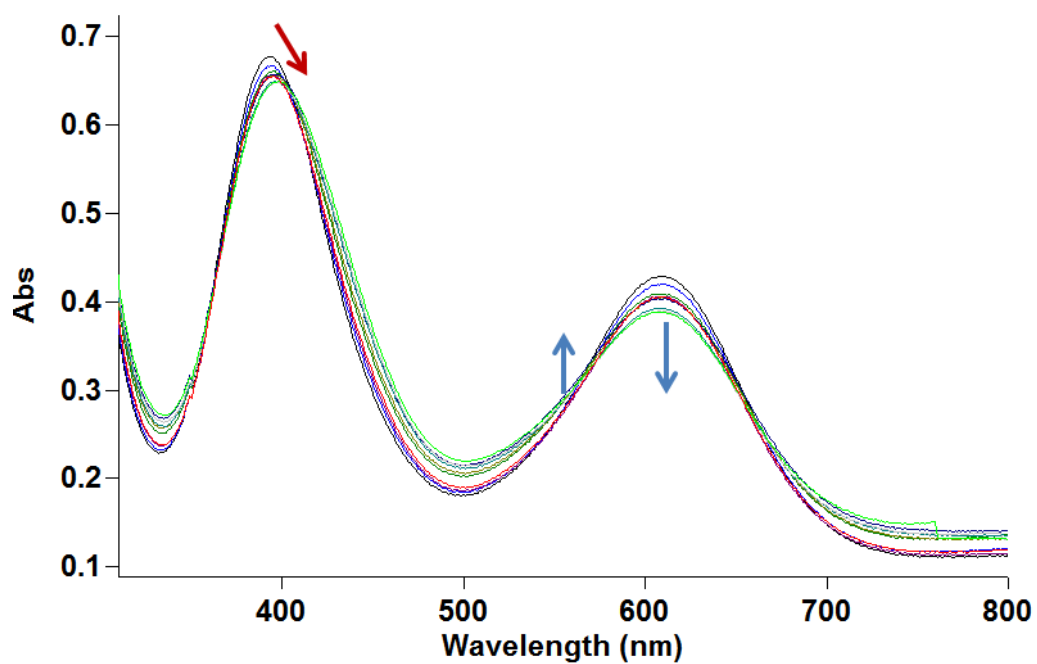


Figure 1. Titration of 250 μ M 5mAz, pH 6.5 KPi, with KBr 10 to 100 mM KBr, 10 mM increments. The 420 nm peak red-shifts, while the 600 nm peak decreases, seemingly in concert with another peak that appears to grow in around 550 nm.

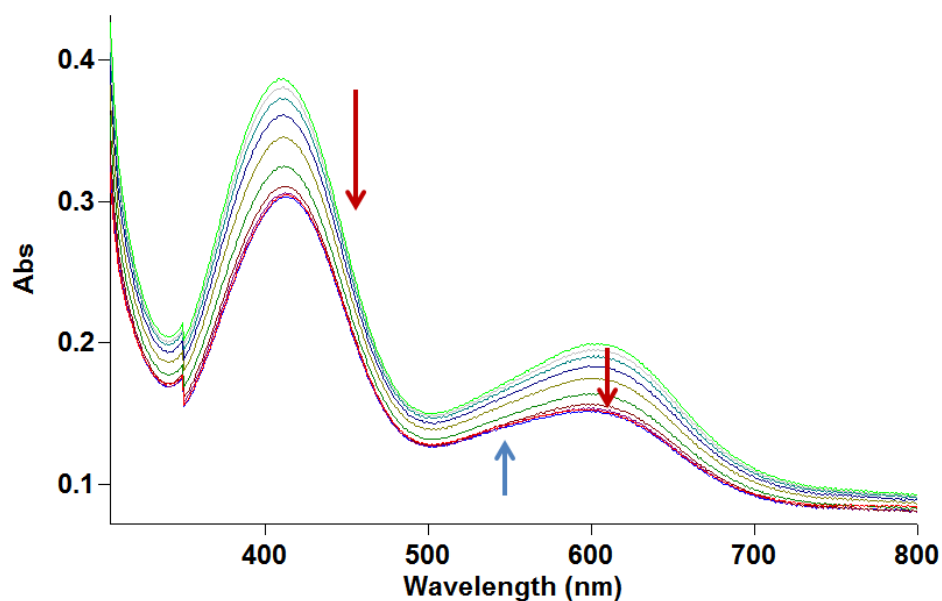


Figure 2. Titration of 250 μ M 5mAz, pH 6.5 KPi, with KBr 100 to 500 mM KBr, 50 mM increments. At higher Br⁻ concentrations, no shifting of peak position is noted, but a 550 nm peak continues to grow in, and the 400 nm peak becomes diminished along with the 600 nm peak.

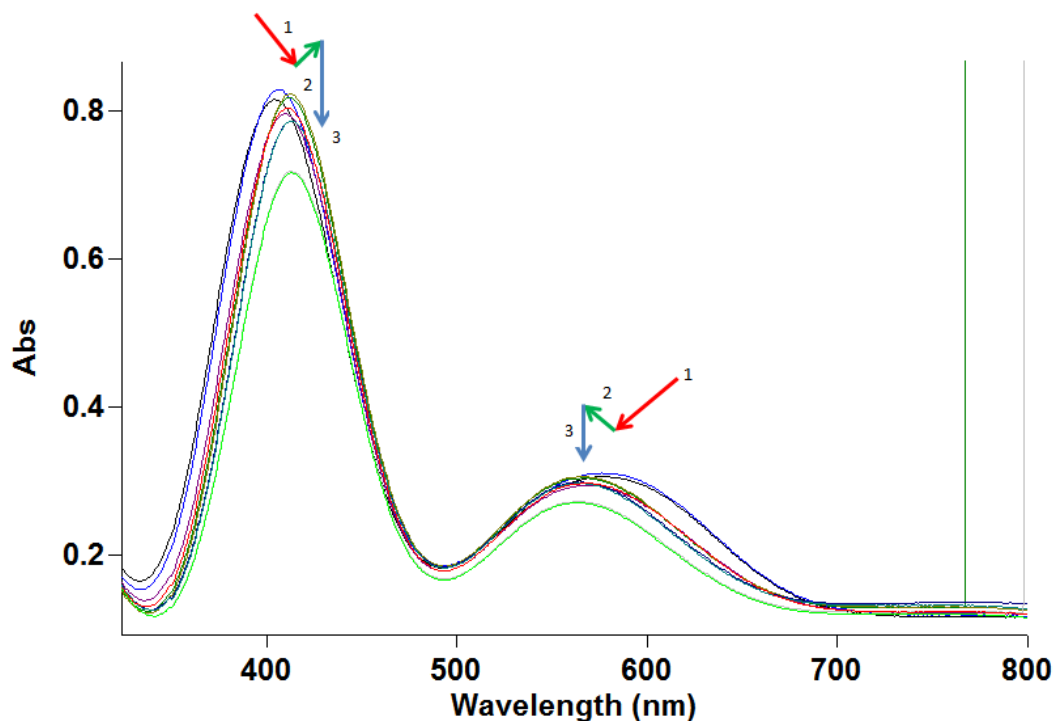


Figure 3. Titration of 250 μ M 5mAz, pH 7 KPi, with aliquots of pH 7.2 imidazole, giving a gradient of imidazole concentration from 0-100 mM in 10 mM steps. The titration exhibits complex visible spectrum changes at both peaks. In the first step, both peaks shift to lower absorption, and towards one another. In the second step, absorption increases slightly (not above A_0), and the peaks continue to shift towards one another slightly. In the final step, absorption decreases dramatically, but no shift in wavelength is observed.

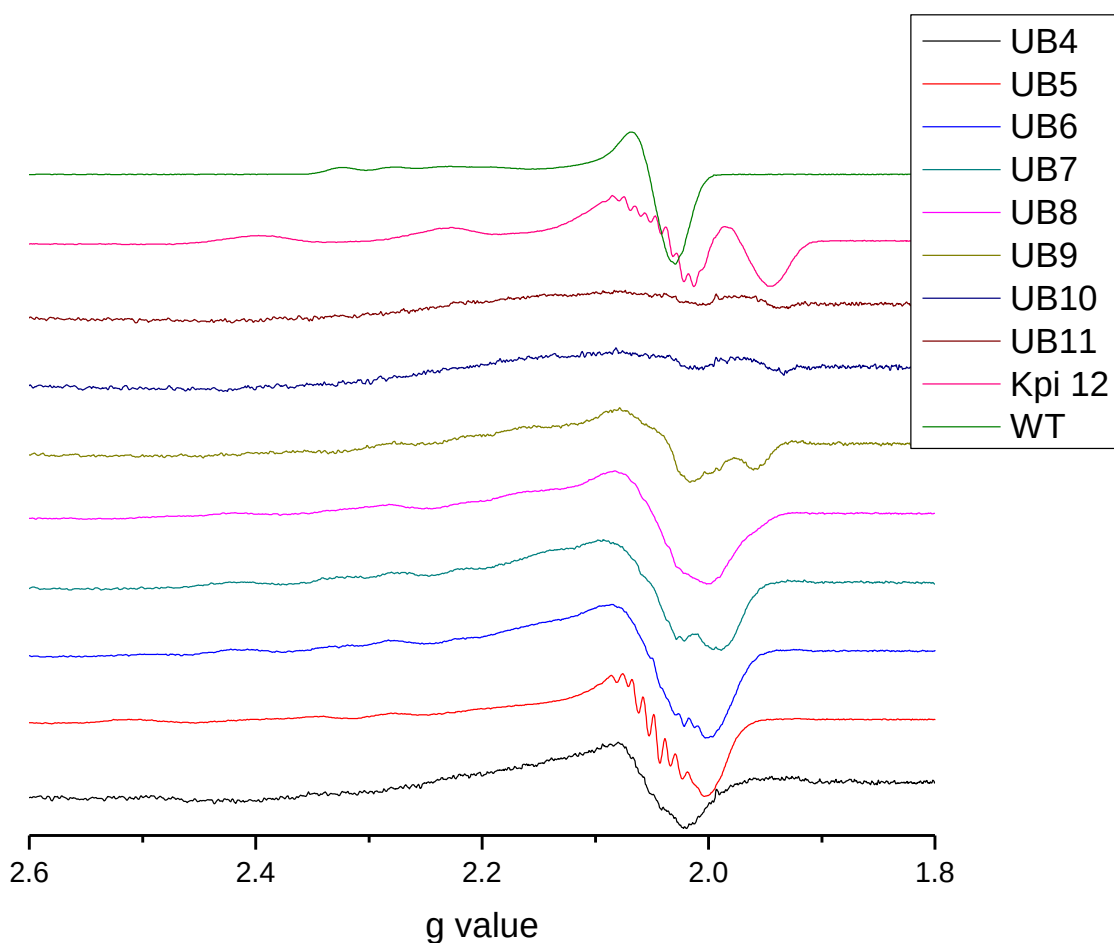


Figure 4. EPR spectra for the 5mAz, showing the wide variety of geometries attained by the protein. Further EPR characterization of each component as separated by size exclusion must be performed, as the current EPR data shows an ensemble of species. It is, nonetheless, helpful in providing an understanding of the behavior of the protein, and provides evidence for site geometry changes in a pH dependent fashion. It also appears to indicate that values between pH 4 and pH 6 provide the most similarity to wild type, while a definite divergence is noticeable after pH 7. At pH 8, it is believed that deprotonated water is now bound directly to the copper atom, indicating an open binding site capable of interacting with solvent and other soluble ligands.

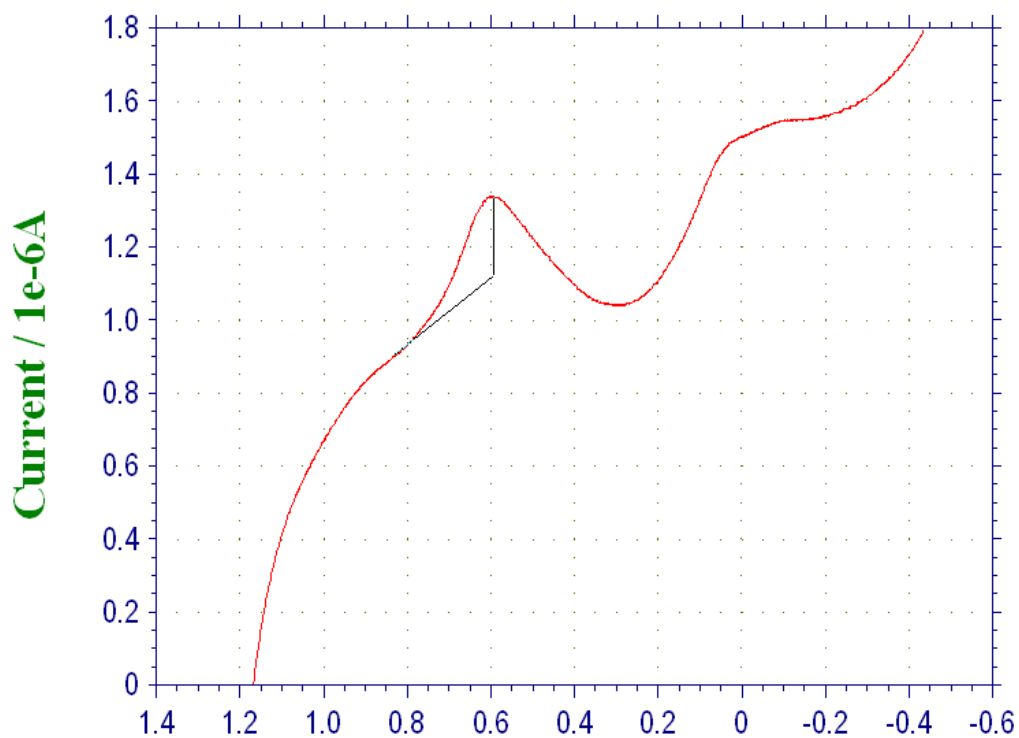


Figure 5. Cyclic voltammetry trace, showing the reductive peak (approximately 597 mV vs. Ag^+/AgCl). Conditions: 10 μL 10 μM 5mAz was added to a PGE electrode coated with graphene oxide (deposited from H_2O). The protein was covered with a dialysis bag and placed in an electrolyte solution of pH 6 ammonium acetate (50mM) buffer

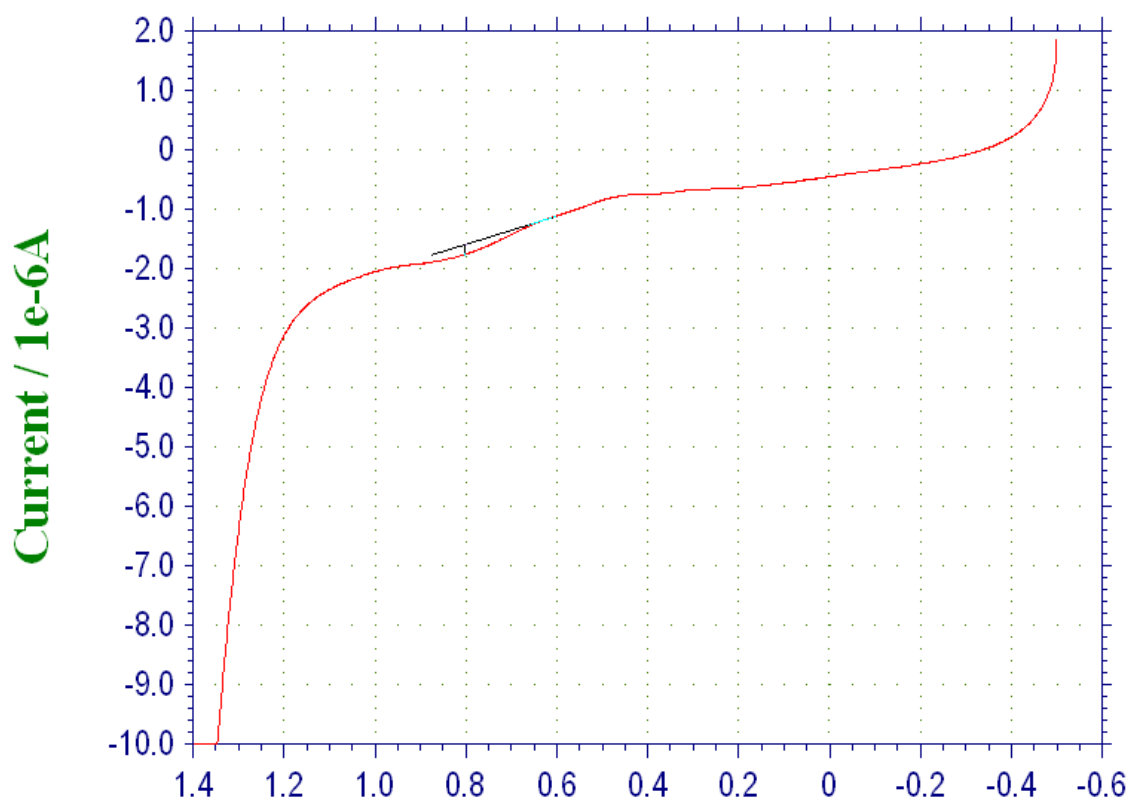


Figure 6. Cyclic voltammetry trace, showing the oxidative peak of the Cu^{2+} titrated 5mAz (approximately 800 mV vs. Ag^+/AgCl). Conditions: 10 μL 10 μM 5mAz was added to a PGE electrode coated with graphene oxide (deposited from H_2O). The protein was covered with a dialysis bag and placed in an electrolyte solution of pH 6 ammonium acetate (50mM) buffer

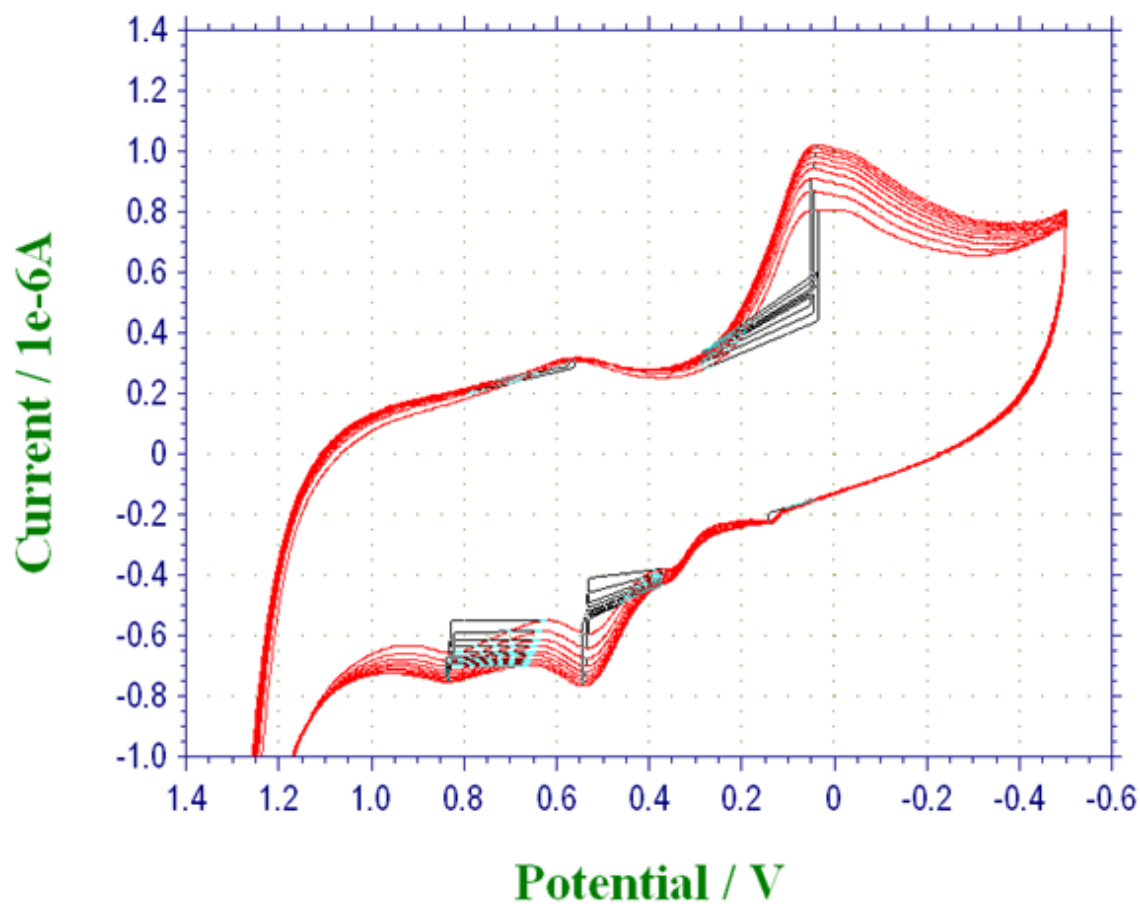


Figure 7. Cyclic voltammetry trace, showing ancillary peaks possibly attributable to oxidative damage of the Cu^{2+} titrated 5mAz through 50 scans. Conditions: 10uL 10 uM 5mAz was added to a PGE electrode coated with graphene oxide (deposited from H_2O). The protein was covered with a dialysis bag and placed in an electrolyte solution of pH 6 ammonium acetate (50mM) buffer

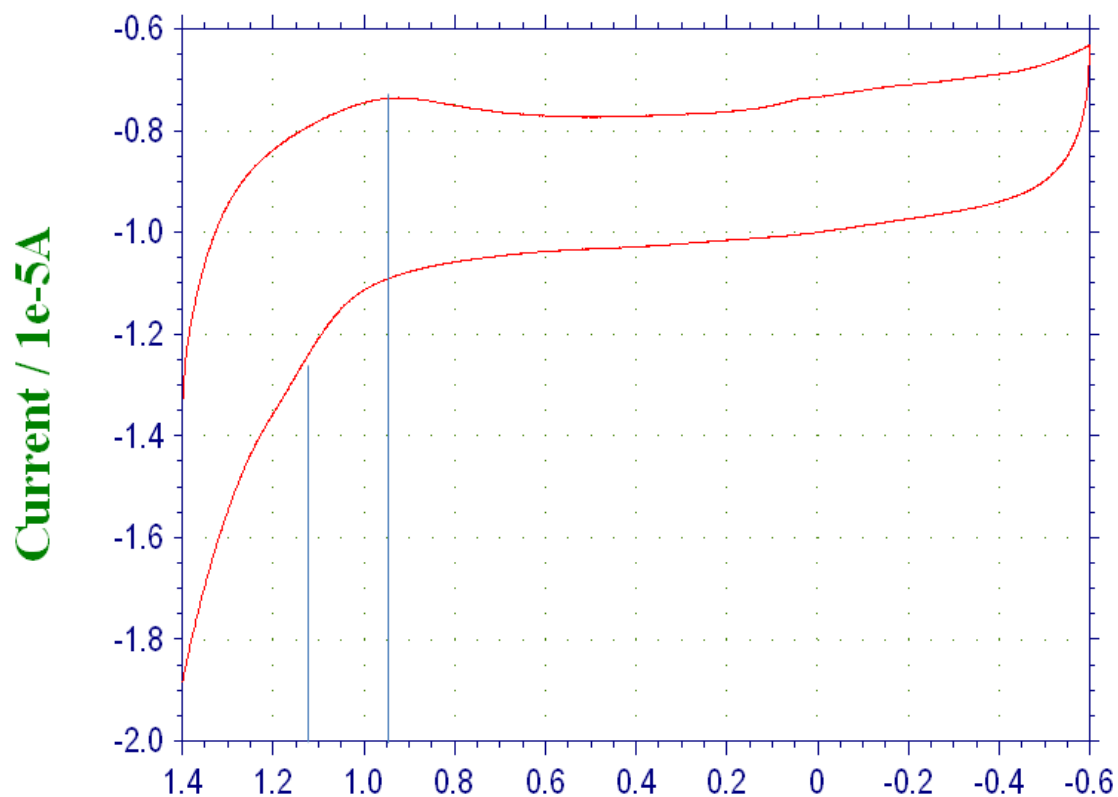


Figure 8. Cyclic voltammetry trace, showing the oxidative and reductive peaks of the Cu^{1+} titrated 5mAz (approximately 1.125 V and 0.95V vs. Ag^+/AgCl , respectively). Conditions: 10uL 10 uM 5mAz was added to a PGE electrode coated with graphene oxide (deposited from H_2O). The protein was covered with a dialysis bag and placed in an electrolyte solution of pH 6 ammonium acetate (50mM) buffer

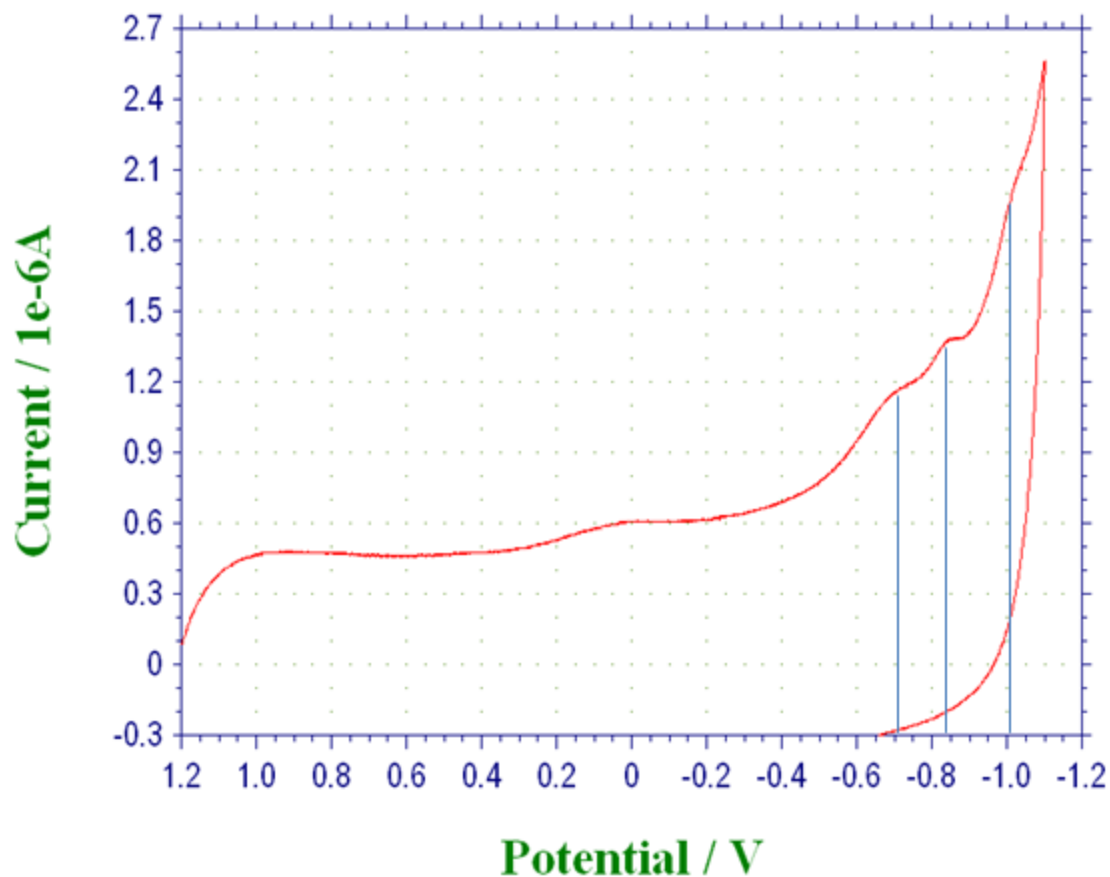


Figure 9. Cyclic voltammetry trace, showing reductive peaks at highly reducing potentials taken after the protein (Cu^{1+} titrated 5mAz) and electrode were degassed in the cell for over 1 hour. Several new peaks arose, at approximately -0.7, -0.85, and -1.0V vs. Ag^+/AgCl . As there are few reducible residues within the protein, this may indicate an unusual oxidation state, and at the least is remarkable in that it would indicate . Conditions: 10uL 10 μM 5mAz was added to a PGE electrode coated with graphene oxide (deposited from H_2O). The protein was covered with a dialysis bag and placed in an electrolyte solution of pH 6 ammonium acetate (50mM) buffer

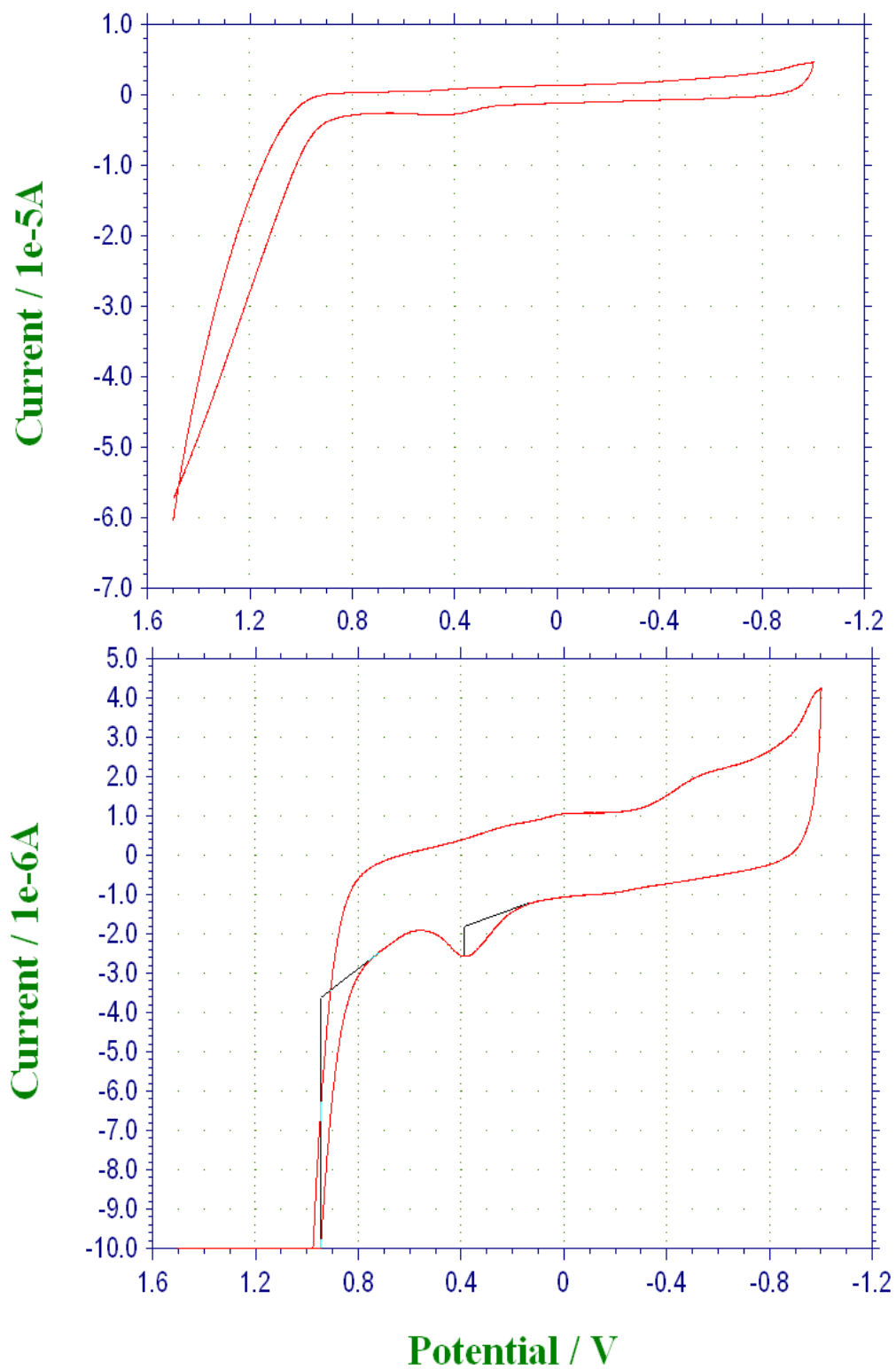


Figure 10. Zoomed and unzoomed scans of 5mAz (Largest eluted peak from size exclusion column, Cu^{2+} titrated). Initial scans show what appears to be a catalytic wave beyond 0.9V vs. Ag^+/AgCl . This may be due to catalytic oxidation of water, ammonia, or another as-of-yet unknown substrate.

CHAPTER 2: EXAMINATION OF THE CATALYTIC ACTIVITY OF THE M44F/N47S/F114N/G116F/M121L AZURIN VARIANT

Introduction

Polymerization of olefins has been a topic of significant research interest for more than 60 years^{10,11}. The first industrial polymerization catalysts include the Ziegler-Natta catalyst, which was the first major industrial catalyst to be capable of tactic polymerization. Modern research catalysts rely on a variety of methods, ranging from free-radical based polymerization (the least expensive method, but the method that also produces polymers of the lowest quality and homogeneity), to lanthanide-based sandwich complexes capable of providing 99%+ tacticity and millions of turnovers^{12,13, 14}. Atom Transfer Radical Polymerization (ATRP) complexes lie somewhere between these two systems, and are of interest for their ability to provide substantial control over polymer size, while at the same time using simple ligands that do not require exotic metals^{15, 16}. In fact, ATRP complexes primarily rely on copper for activity, a characteristic shared between these catalysts and the protein investigated in this thesis, Azurin. The drawback to these simpler catalysts is a lack of direct control over tacticity, as well as limitations on the means of control over chain size.

Proteins exist that bind copper with the ability to exquisitely tune the properties of the copper atom, and perform a variety of catalytic activities. Indeed, olefin and conductive polymer generation has been reported before, albeit using a completely different class of proteins - heme peroxidases – and an indirect route to polymerization relying on the generation of free radicals¹². In the case of these proteins, hydrogen peroxide is added to produce a highly reactive intermediate known as compound I, a radical cation and ferryl iron within a heme cofactor. The net effect is a very potent oxidant that can transfer a radical throughout the

protein via redox-active amino acids, and thus to a solvent or substrate. Just as importantly, many of these catalytically active proteins can have potent control over stereochemistry due to the structural features of their active sites. A combination of the control of protein catalysts with more efficient synthetic polymerization catalysts may enable us to perform polymerization chemistries with novel control over polymer secondary structure, as well as opening up polymerizations using monomers whose reactions have not been efficiently catalyzed by conventional catalysts.

Due to its ready availability and decades of thorough characterization, as well as its relatively high yield, we have chosen to use the small cupredoxin Azurin as a possible candidate for a protein olefin polymerization catalyst. A mutant of this cupredoxin has been developed with an unprecedentedly high reduction potential of almost 1 volt versus NHE. This mutant (M44F/N47S/F114N/G116F/M121L *Pseudomonas aeruginosa* Azurin) is an unusual green copper protein, with absorbances dominating at 420 and 600 nm. Extensive characterization has lead us to believe that this cupredoxin may exist with an open binding site on the copper ion cofactor, or with a histidine ligand (H117) that exists in a state of bound/unbound flux (see chapter 1). As a result, novel catalysis may occur that takes advantage of both the electron transfer and catalytic properties of such a protein. This may make for an aqueous-solution polymerization catalyst which appears to be capable of polymerizing a variety of olefins with some modicum of control over their secondary structure.

Materials and Methods

Preparation of *P. aeruginosa* Azurin

Azurin variant M44F/N47S/F114N/G116F/M121L was prepared via expression in *E. coli*. Cells were transformed with the Azurin-containing pET9a vector with the appropriate mutations to the gene sequence and were selected for using Kanamycin when plated. Overexpression was accomplished in BL21DE3 STAR cells from Invitrogen. Overnight cultures of the BL21DE3* transformation were prepared by inoculation of 5 mL LB (Lysogeny Broth) incubated at 37C, containing 100 ug/mL Kanamycin. After overnight incubation, 5 mL LB culture was added to 2L 2xYT (32g Bacto-Tryptone, 20g Yeast Extract, 10g NaCl) broth in 6L flasks. Cultures were grown at 37C, shaking at 210 RPM, until an OD₆₀₀ of 1.5 or more was reached, followed by a period of 4-5 hours of induction using approximately 200mg/L IPTG at 37C. After the induction period, cells were harvested by centrifugation in 450 mL centrifuge bottles at 8000 RPM (ca. 10,000xg) for 10 minutes. Cells were then resuspended in their centrifuge bottles using a total of 1L sucrose solution (20% sucrose, 0.5g EDTA, 5g TRIS-Base, adjusted to pH 8 with HCl; 150 mL per bottle), and shaken in the solution for 1 hour at room temperature. Cells were re-pelleted at 10,000xg for 10 minutes, and 1L osmotic shock solution (0.98g NaCl, 4 mM DTT, chilled to 4C) was added to the pellets to rupture the periplasmic membrane. Cells were shaken with the osmotic shock solution at 4C for 10 minutes, and were subsequently re-pelleted (10 minutes, 10,000xg). The supernatant was collected, and contaminants precipitated from the supernatant using a volume of precipitation solution (400mM NaOAc, pH 4.0) equivalent to 10% of the osmotic shock supernatant. After precipitation, cells were once again centrifuged (10 minutes, 10,000xg). The resulting supernatant was collected, and incubated with SP-sepharose beads (GE Healthcare) equilibrated with pH 4.1 Ammonium acetate for approximately 2 hours under

gentle shaking. The beads were then added to an FPLC column, and a customized gradient was run on an Akta FPLC (GE Healthcare) to remove further impurities. The gradient was composed of a 2-buffer system: 50 mM Ammonium Acetate – Buffer A: pH 4.1; Buffer B: pH 6.35. A step gradient was performed with 3 steps – 55%B step to remove impurities, and a subsequent 100%B step to elute the partially purified Azurin, which was then kept for further purification. The eluted Azurin was then concentrated and further purified using a Q sepharose column (method: column equilibrated with 100% buffer B, causing protein to run through while contaminants were retained on the column). In the final purification step, a size exclusion column (S-100 beads, GE healthcare) was used to separate different variants of the mutant as well as remaining contaminant protein.

Azurin Titration with Cu²⁺

Azurin was titrated with Cu²⁺ or Cu¹⁺ according to experimental need. Copper was added slowly and in 1/10th equivalents (calculated using $\text{Epsilon}_{280} = 9800\text{mM}^{-1}$), on ice and with rapid stirring in a glass test tube, and with 3-5 minutes between additions. The absorbance at 420 nm and 600 nm was monitored between each addition, with the ratio between the 600 nm Cu-S MLCT band and Tryptophan 280 nm absorbance monitored in order to determine when saturation binding has been reached (R/Z between 5 and 10 depending on quality of the protein).

Styrene Polymerization

Styrene polymerization was tested under a variety of conditions in an attempt to optimize the polymerization reaction. The most effective conditions found utilized an external oxidant to promote turnover of the Azurin catalyst – K₂IrCl₆ was used at pH 8, in 50 mM NaPhosphate buffer. 5 mg Iridium Chloride was added to a 1 mM solution of 5mAz

(approximately 1 mL) exchanged into phosphate buffer. To this solution, 5 mL styrene monomer was added, and the resulting mixture was vortexed for 3 minutes, and then allowed to sit for 1 week. The resulting material was lyophilized, and was collected into a glass vial for characterization via DSC. Other reactions using iridium chloride as an oxidant and no oxidant were performed in a variety of screens – using K_2IrCl_6 was found to produce what is believed to be styrene polymer at the greatest rate (significant amounts of polymer could be recovered after only 10-15 minutes). While 5-mutant Azurin on its own was able to produce styrene polymer, the protein on its own required substantially more time than in the presence of Iridium Chloride oxidant. A positive control (styrene, MW: 10,000) was used for comparison, and melted at approximately 114°C

EDOT Polymerization

5mAz was also tested with the monomer EDOT for possible polymerization activity. EDOT monomer was mixed with Azurin (250 uM, pH 6.0) at a variety of ratios with the amount of monomer kept constant. 100uL-1mL protein was mixed with 200 uL EDOT, and was allowed to incubate at 45-50C for 1 week. It was found that a ratio of 800uL protein:200uL EDOT appeared to be most effective, particularly when exposed to air. The EDOT screen was repeated with low quantities of monomer (100-200 uL) in the presence of high concentrations of protein (2 mM). 100-500 uL of the 2mM protein solution was mixed with the monomer at a variety of pH values, with the most important results being found at pH 4 and pH 8. pH 4 yielded a glassy solid, likely with a high hardness factor, as the solid produced an audible scraping noise when pressed against glass. pH 8 produced a dark brown solid in high quantities compared to the starting volume of EDOT – as high as 80% yield was observed. Polymers were examined using DSC.

Pyrrole Polymerization

Polymerization of Pyrrole was carried out in a manner similar to EDOT and Aniline. 2000 uL monomer was added to a variety of protein solution volumes ranging from 100-1000uL of 250 uM 5mAz. A black solid was formed in mixtures with a low volume of protein. Further screens using high 5-mutant Azurin concentrations yielded larger quantities of the black solid: a volume of pyrrole (100-500 uL) was mixed with a volume of 5-mutant azurin at a concentration of 2 mM in acetate buffer (pH 6.3). A dark brown solid appeared within 5 minutes in the pyrrole layer, and was allowed to mature over 2 days. The resulting sample was collected, dried, and examined using DSC.

Characterization of Styrene Polymerization product via DSC

The product formed from the most active styrene polymerization reaction was characterized via DSC, using a gradient from 50C-400°C (50°C/min). The DSC melt chamber was cleaned between samples using a 25-600°C gradient, holding the chamber at 600°C to degrade and vaporize all contaminants.

Results

Several polymers appear to have been successfully produced using only 5-mutant Azurin and (if needed) an assisting oxidant. Styrene, the most difficult of the polymers tested to polymerize due to its poor interaction with the protein (poor water solubility, poor active site access), was successfully synthesized in sufficient quantity for DSC using Iridium Chloride as an oxidative regenerator of the catalyst (**Figure 11, top**). The most promising resultant product appears to have two melting points (**Figure 12**), one of which appears at 169.33°C, and the other at 297°C – in literature, these high melting point products (when compared with the low control styrene melting point) are often attributed to the presence of isotactic and syndiotactic styrene,

respectively⁵. The inordinately high melting point of this may be due to the presence of Iridium chloride and protein in the sample, which may have cross linked with the polymer.

Pyrrole is by far the most responsive monomer to the 5-mutant variant, showing significant material that can be ascribed to growing polymer within only a few minutes. The melting point properties of this material as well appear to indicate a doped polymer structure – significantly broadened melting point peaks are noted to be due to the presence of a dopant in conductive polymers like this. Low Azurin concentrations give well defined melting point peaks (**Figure 13**) at 120 and 160°C, while higher concentrations and ratios favoring the protein give significantly broadened peaks (**Figure 14**). Lastly, the conductive polymer EDOT gives a very interesting, almost crystalline or glassy product with a very well defined melting point (**Figure 15**), set at 260°C. This would seem to indicate that the protein is not interacting with the structure of the EDOT polymer, which is to be expected given its amphiphobic nature.

Discussion

“5-mutant” (M44F/N47S/F114N/G116F/M121L) Azurin has been shown to be able to produce what appears to be tactic polystyrene at pH 8. We believe this product may be doped tactic polystyrene based on its unusually high melting point, and the presence of both Iridium Chloride and Azurin, both of which could act as doping agents. Unfortunately, dissolving the product has proven elusive as of this time – even testing a variety of high boiling point solvents (dichloro/trichlorobenzene, which have been used to dissolve tactic pS in the past) yields little dissolution, making characterization using techniques such as NMR and GPC difficult. For this reason, other monomers have become the focal point of this project.

Conductive polymers are of significant importance in the production of modern electronics, in particular of 3,4-ethylenedioxythiophene (PEDOT)^{17,18,19,20}, and pyrrole^{21,22,23,24,25,22}. These polymers have been used in the production of transparent electrodes and electrochromic devices, as well as in the production of electrodes for interface with biological systems. Other conductive polymers of importance (aniline, thiophene) have been tested, but have not proven tractable to the protein thus far. Because EDOT and pyrrole require a dopant for conductivity, it is our hope that polymers impregnated with the 5-mutant (and perhaps other Azurin variants better suited to electron transfer) may become conductive, opening interesting new avenues into the modification of polymers using Azurin derivatives with unusual redox properties. Current industrial polymerization methods for these polymers rely on polymerization at electrodes, using simple catalysts such as iron chloride (FeCl₃) in aqueous solution, and in the case of EDOT, using copolymers like polystyrenesulfonate to improve the solubility of the final product. Using a protein in place of these more conventional catalysts may allow for the manipulation of the properties of the final polymer product via manipulation of the protein sequence. We have generated what we believe to be polystyrene, polypyrrole, and poly-EDOT in aqueous solution at room or slightly elevated temperatures, in oxygen, and, in the case of pyrrole, at high rates. The use of DSC to characterize these polymers shows very unusual melting points as compared to what is reported in literature. In the future, the electrical properties of these conductive polymers will be examined. As Azurin is a potent electron transfer catalyst, it is our hope that its inclusion in a resulting polymer will improve its properties.

Chapter 2: Figures



Figure 11. Physical appearance of two different 5-mutant Azurin-generated polymers. **On Top:** Styrene only (left) and Styrene+Iridium Chloride (50 equivalents, right). **On Bottom:** Pyrrole polymerization, showing the rapid clouding and thickening that takes place immediately after pyrrole is added, even without mixing. Conditions: 0.5 mL pyrrole is added to 0.5 mL 250 μ M 5-mutant Azurin. A cloudy, dark, gooey layer has formed within 5 minutes of addition in the top (pyrrole) layer. With additional time, this layer will turn to black.

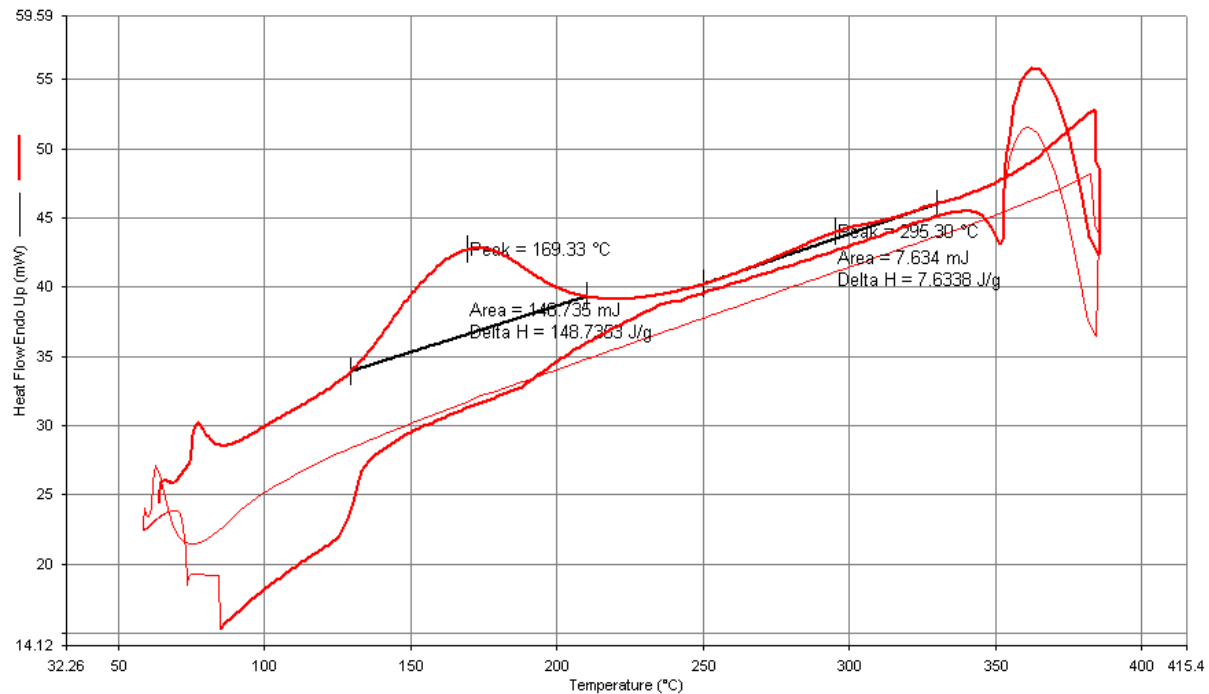


Figure 12. DSC scan spectrum for the styrene polymerization product of 5mAz incubated with K_2IrCl_6 at pH 8 in phosphate buffer. Peaks are noted at 169.33C and 296.3C – the exceptionally high melting point of this polymer may be due to the protein and high concentration of iridium chloride, which may have affected the melting point of any tactic pS.

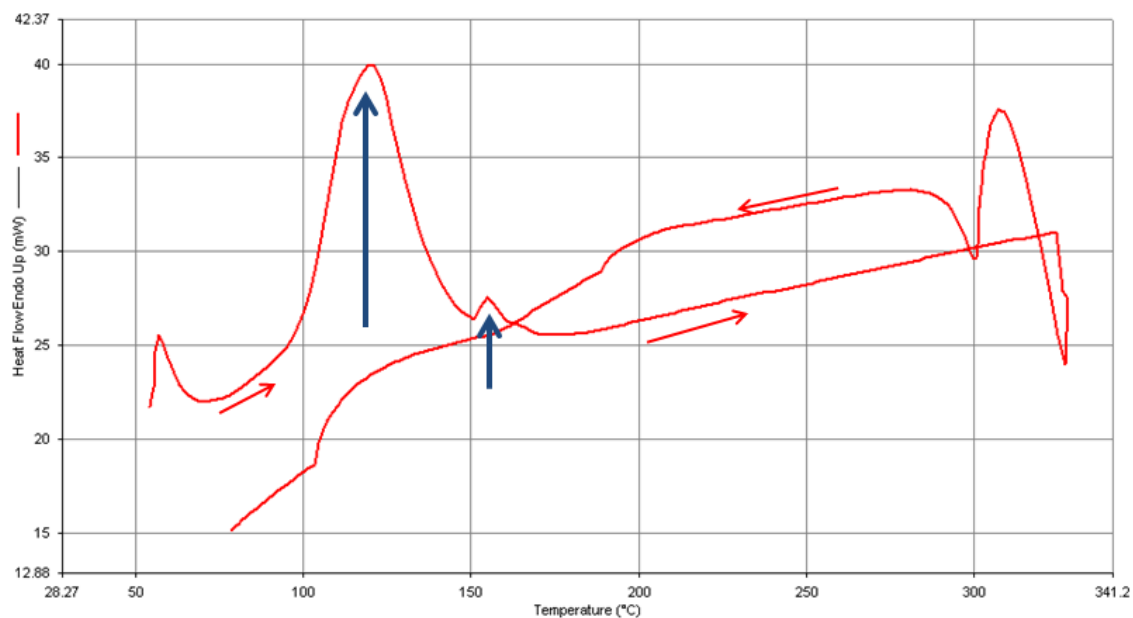


Figure 13. Polypyrrole synthesized using 5-mutant Azurin (MP: 120C, with a second minor mp at 160C). 0.1 mL 5-mutant Azurin was added to 0.25 mL pyrrole, and was incubated at 50C for 2 weeks. The result was a black goo. Literature descriptions of pyrrole are “a black solid.” Blue arrows indicate melting point peaks, while red arrows indicate heating or cooling.

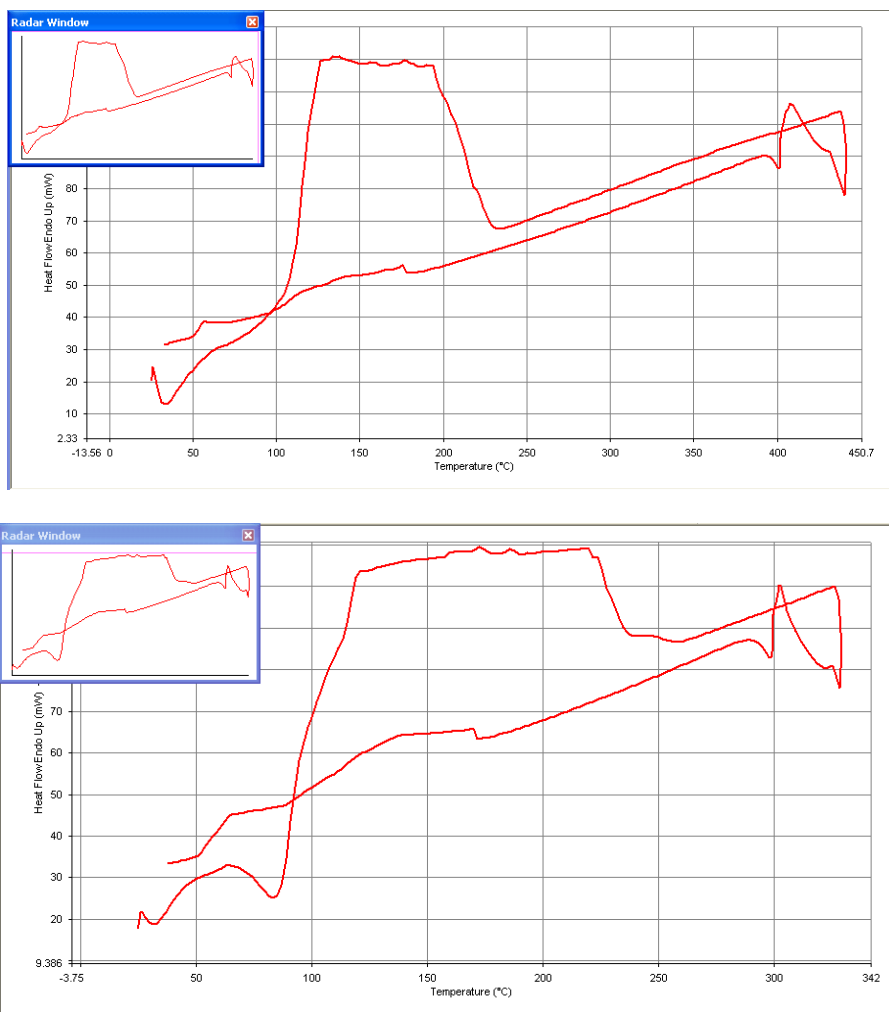


Figure 14. This figure shows ratios of 500 uL pyrrole to 500 uL 5mAz (**top**, “melting point” between 125 and 190C), and 500 uL pyrrole to 400 uL 5mAz (**bottom**, “melting point” between 120 and 225C). While this exceptionally broad peak may appear to be an artifact, the instrument has not reached its detection limit by any means – rather, the heat release profile for these mixtures is quite unusual, and may be indicative of a doped polymer structure. Further characterization is necessary to understand the nature of this polymer.

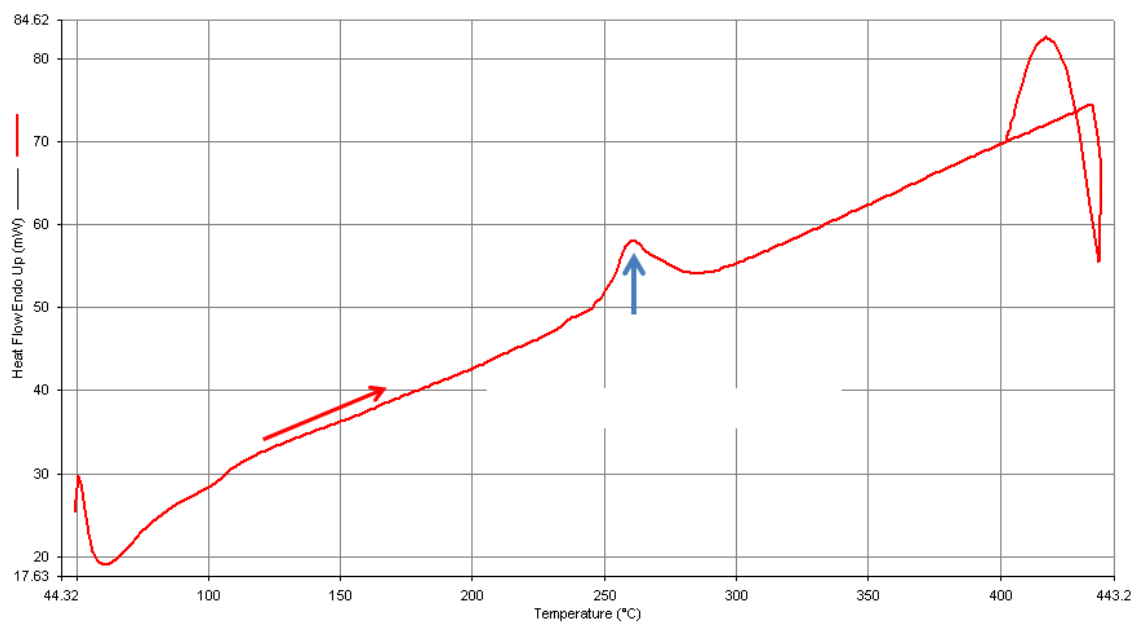


Figure 15. EDOT, on reaction with 5mAz, forms an almost glassy polymer material, which exhibits a melting point around 260C. 200 uL EDOT was added to 300 uL 5mAz (2mM), pH 4.0 NH_4OAc (50 mM). The mixture was incubated at 50C for 1 week.

CHAPTER 3: PRODUCTION OF A TRIMERIC LACCASE (SLAC FROM *Streptomyces coelicolor*) USING A CODON-OPTIMIZED SYNTHETIC GENE.

Introduction

Laccase is comprised of several electron transfer and catalysis cupredoxin sites – a type I (mono-copper; electron transfer), type II (mono-copper; catalytic), and type III (di-copper; electron transfer/catalysis)^{30,31}. Via the manipulation of secondary-coordination sphere characteristics surrounding these sites, we may be able to improve upon the natural oxygenase activity of *Streptomyces coelicolor* Laccase (SLAC, or small laccase), and improve the communication of electrons between the active site of this protein and the electrode to which it is bound. Without a dramatic improvement in the current density that can be generated at biological half cells that perform the reaction $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$, these biofuel cells will not be able to effectively compete with precious metal catalysts in the near future. This particular laccase is of interest for its improved rate of oxygen reduction as compared to other laccase variants studied in the past^{32,33}. Unique features of this enzyme (a trimer with active sites present at the interface of monomers) may be the cause of this increased activity, as the activity of each site is increased as compared to laccases adapted to substrate, and a smaller overall size per active unit should allow for a greater accumulation of current density. Along with the composition of its active sites and electron transfer sites (all cupredoxins), these characteristics make SLAC and laccases more broadly of great interest for their potential in bioenergy as well as test beds to prove the utility of secondary coordination sphere manipulation in modifying enzyme activity via the tuning of redox potential and possibly in controlling electron transfer. In the future, we believe that manipulating the potential of the type I and II sites of this protein to

increase their reduction potential could result in increased rates of oxygen reduction. However, for the time being, we have focused on the cloning and purification of this enzyme from a synthesized gene.

Materials and Methods

Gene Synthesis and Plasmid Amplification. Total gene synthesis and initial cloning was performed for our lab by GenScript. A wild-type gene was submitted that included the twin-arginine-targeted leader sequence, which was then codon optimized for high-level expression in *E. coli* using proprietary software at GenScript. The synthesized gene was inserted into the expression plasmid pGS21a as well as the cloning plasmid pUC57. Both genes were redissolved in syringe-filtered Millipore water to 100ng/mL upon their receipt and were transformed into XL1 Blu cells for amplification. The transformation was accomplished using commercially-made competent XL1 blu cells that were resuscitated using SOC media (45s heat shock at 45C, followed by 1 hour recovery at 37C), and subsequently plated on ampicillin selection media. A colony was collected from this plate, and was grown in 5 mL LB media at 37C for 8 hours. After this growth period, cells were pelleted at 5000 RPM and were mini-prepped using a Qiagen miniprep kit. The resulting pUC57 plasmid was stored, while the pGS21a plasmid was transformed into BL21/DE3 cells for protein expression.

Protein Expression and Purification. A 5 mL culture in LB media containing ampicillin was allowed to grow overnight, and 1 mL was added to 2L 2xYT in a 6L flask, which contained 100 mg/L ampicillin. After 18 hours of growth at 25C, a period of 6 hours of induction using 200mg/L IPTG was used to induce protein production. These conditions were varied to attempt to increase yield, but no verified trend has emerged thus far to allow for the prediction of yield. After induction, cells were collected by centrifugation at 8000 RPM (about 1000xG) using a

FiberLite rotor and Beckman centrifuge. Purification was then accomplished according to the method used by Machczynski et al. The pellet was resuspended in 75 mL/L 10 mM KPi buffer at pH 7.0. Lysis of the cells was accomplished by sonication for 15 minutes. The lysate was then spun down at 20,000xG for 20 minutes, and the lysate collected. 1mM Cu²⁺ was added over the course of 30 minutes in 6 aliquots under stirring to prevent precipitation. After the addition of copper, DNAase I (150 ug) and RNAase (10 ug) were added, and the solution was stirred on ice for 3 hours. The resulting product was then dialyzed against 5L 10 mM phosphate buffer at pH 7 once for 3 hours, followed by 5L buffer containing 1 mM EDTA to chelate excess copper, and 2 more subsequent dialyses in phosphate buffer only. The dialyzed lysate was collected, and was then bound to a DEAE column cleaned using 1M NaCl and equilibrated in the same buffer as that used for dialysis of the protein. An AKTA FPLC was then used for column chromatography, with an NaCl gradient (0-1M NaCl) used to elute the protein. A variety of gradients have been tested, though the protein does not appear to consistently elute at a particular point in the gradient. A step gradient currently appears to have the greatest potential for purification, though the method is still being refined.

Detection of activity using DOPA. L-DOPA was used to detect laccase activity. As has been previously reported by Machczynski et al., activity is retained by this enzyme in PAGE gels that do not use a high temperature denaturing step. The protein was added to a native PAGE gel (TRIS/Glycine) in pH 9.4 TRIS/0.1M Glycine and SDS with Laemmli dye, and was run as normal. A saturated solution of DOPA in 50 mM pH 9.4 TRIS/0.1M glycine buffer containing SDS was used to stain the page gel, by allowing the run native gel to sit in this solution overnight. A brownish band appeared due to the conversion of DOPA to DOPachrome/melanin by SLAC that was easily visible to the naked eye. Native gels did not use SDS in their running buffer.

Results

While we have yet to obtain a high purity laccase sample, we have shown that activity is present within the samples we have thus far obtained. Though not all samples show substantial activity, even our low yield purifications thus far have shown substantial activity as determined from native and PAGE gels that both show DOPA oxidation activity (**Figure 16**). Likely because of the region of the gradient in which the shown samples were collected (1M NaCl), substantial precipitation was observed. Interestingly, this precipitate retained activity when redissolved using detergent. As well, more than one band showed evidence of activity, in a significant departure from the observation of activity in Machczynski et al., wherein only one band showed activity.

Discussion

Protein Purification/Production. Modifying the oxygenation of the culture will likely vary protein production substantially, and changing the temperatures used in production may greatly affect the output product. We have used a constant 25C growth and production temperature, while Machczynski et al. report using a 30C growth and a 25C induction temperature. As well, we have been using 2L 2xYT in a 6L flask, while Machczynski et al. report using 3L in an unspecified volume flask. Manipulating these variables, as well as other variables such as Cu^{2+} concentration may substantially improve our product yield. As well, improving our FPLC purification technique could improve purity greatly – Machczynski et al. report 97% purity with only a single DEAE column run using an identical gradient. It is clear that we must adjust our purification technique, given the difficulty we have had in obtaining pure protein. As well, modifications to the sequence of the synthesized gene may prove necessary in effective

production of this protein, as particular stall points in translation may not have been retained in the codon optimization of the gene as performed by GenScript.

Mutations. Improving communication between the electrode and this protein is essential to improving its utility as a fuel cell half-cell. Creating a string of tryptophan residues leading to the linker that attaches this protein to the electrode may increase electron transfer rate to the type I site, assuming its reduction potential is adequately high, while engineering the type I and type II sites to increase reduction potential by manipulating the hydrogen bonding networks near these residues could also help increase electron transfer. Altering the secondary coordination sphere ligands that control the structure of the trinuclear active site has tremendous potential to vary the activity of this laccase, though obstacles remain in the alteration of this active site, as the formation of multimers is essential for activity. Improving this interface using computational methods (i.e. Rosetta) and covalent linkages (disulfide bonds) could circumvent this issue.

Chapter 3: Figures

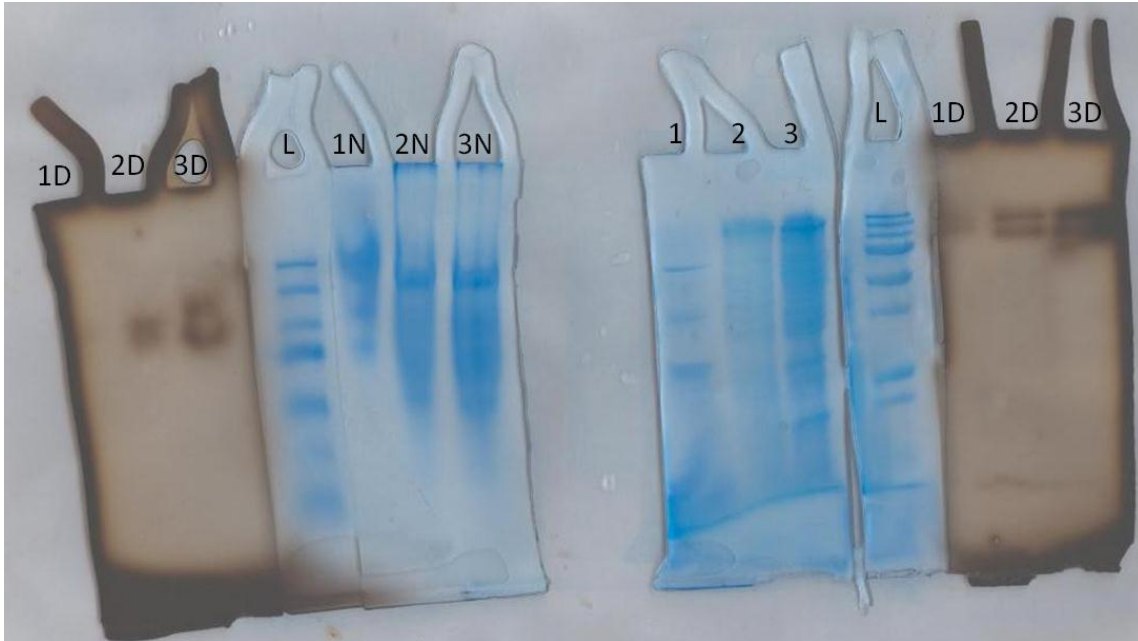


Figure 16. Gels showing DOPA oxidation activity by laccase present in protein purification.

Lanes in the native gels (1N, 2N, 3N) and semi-denaturing (no heat) gels (1,2,3) match with lanes in the respective DOPA stain gels to which they are adjacent. Lane 1 is protein that did not bind the DEAE column, while lane 2 and lane 3 represent both precipitate and solute in the blue-green fraction.

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