Amy Gao is a resident of the beautiful Washington State. Growing up around the natural paradox of water and desert, she lives for nature and loves contradictions. As a child, she vastly enjoyed running between the evergreen trees with her beloved twin brother, letting their imaginations roam free. Now, Amy is studying mechanical engineering at Olin College in Needham, MA, and hopes to earn her Ph.D. in engineering one day. She held one SULI internship at Pacific Northwest National Laboratory in the summer of 2007. Amy likes chocolate, adventuring, design, running, and all things bright and shiny.

Robert Heck is a senior research scientist at the Pacific Northwest National Laboratories (PNNL). He received his Ph.D. from the University of Florida in 1996 for a study of the structure and function of murine carbonic anhydrase isozyme V. His postdoctoral research at the University of Pennsylvania focused on using xray crystallography to explore carbonic anhydrase isozymes and several other proteins. His research activities as PNNL have focused on using solid state NMR techniques to investigate the structure and function of zinc, magnesium, copper, and calcium metalloproteins.

Large Scale Production, Purification, and ^{65}Cu Solid State NMR of Azurin

Amy Gao and Robert W. Heck

ABSTRACT

This paper details a way to produce azurin with an efficiency over 10 times greater than previously described and demonstrates the first solid state nuclear magnetic resonance spectrum of ^{65}Cu(I) in a metalloprotein. A synthetic gene for azurin based upon the DNA sequence from *Pseudomonas aeruginosa* including the periplasmic targeting sequence was subcloned into a T7 overexpression vector to create the plasmid pGS-azurin, which was transformed into BL21 (DE3) competent cells. The leader sequence on the expressed protein causes it to be exported to the periplasmic space of *Escherichia coli*. Bacteria grown in a fermentation unit were induced to overexpress the azurin, which was subsequently purified through an endosmotic shock procedure followed by high performance liquid chromatography (HPLC). 1,500 mg of azurin were purified per liter of culture. ^{65}Cu(II) was added to apo-azurin and then reduced. The ^{65}Cu metal cofactor in azurin was observed with solid state nuclear magnetic resonance (NMR) to determine any structural variations that accompanied copper reduction. This is the first solid state NMR spectra of a copper(I) metalloprotein. Analysis of the NMR spectra is being used to complement hypotheses set forth by x-ray diffraction and computational calculations of electron transfer mechanisms in azurin.

Introduction

Pseudomonas aeruginosa has been shown to be highly cytotoxic to certain cancer cells. It produces the protein azurin in the presence of cancer cells, which stabilizes the tumor suppressor p53, inducing apoptosis [1]. However, *P. aeruginosa* is an opportunistic bacterium that is often lethal to immunosuppressed patients, including those with AIDS, cystic fibrosis, severe burns, and cancer. The bacterium thwarts cancer from claiming the life of the patient, but it may live on as a parasite, ultimately killing the patient. However, it is the protein azurin, produced by *P. aeruginosa*, not the bacterium itself, that cancerous cells find deadly. Previous reports show up to 100 mg of recombinant azurin have been produced per liter of culture [2]. With slight alterations to a method provided by Karlsson *et al.* [3], we found that the yield can be increased over tenfold. These methods are described in this paper.

The structure and function of the redox copper metalloprotein azurin has been studied extensively by redox biochemistry, x-ray diffraction, and computational approaches. Azurin is a 14-kilidalton (kDa) protein, composed of a beta barrel backed by an alpha helix. It is a blue-copper metalloprotein characterized by a deep azure hue when Cu(II) is bound to the protein by its three planar trigonal ligands — two histidines and a cysteine. An axial methionine and an axial glycine backbone carbonyl complete the trigonal bipyramidal ligand sphere around the copper atom [4] (Figure 1).

The reduced copper azurin has been shown to have a similar structure to the oxidized form by Crane *et al.* [4] (Figure 2), but the postulated changes in copper-ligand bond lengths and angles are less than the experimental errors of x-ray crystallographic structure determination for the oxidized and reduced forms. The ability of azurin to lose and gain electrons gives it the role of an electron-transfer protein in *P. aeruginosa*, possibly one that donates electrons to nitrite reductase under stress. The redox reactions cause some slight structural changes in the protein but cause a large change in the visible absorption spectrum of the bound copper ion. Although much is known about the chemical environment around the copper in oxidized azurin, solid state NMR of ^{65}Cu provides some valuable new insights into the previously spectroscopically silent reduced form of azurin.

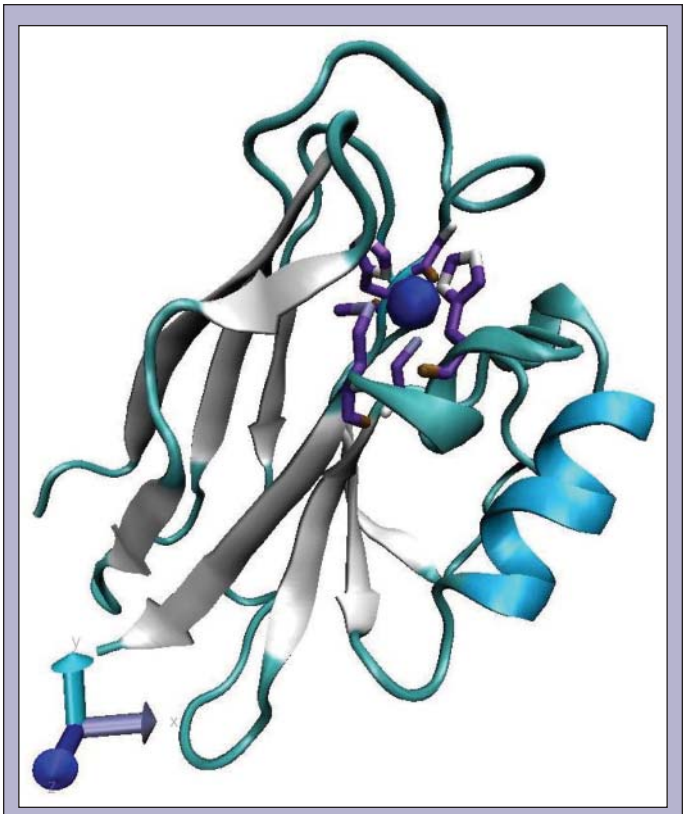

Figure 1. A structural model of the oxidized-copper azurin molecule. The copper ion is bound by his 46 and 117, cys 112, met 121, and gly 45. (Crane et. al., 2001.)

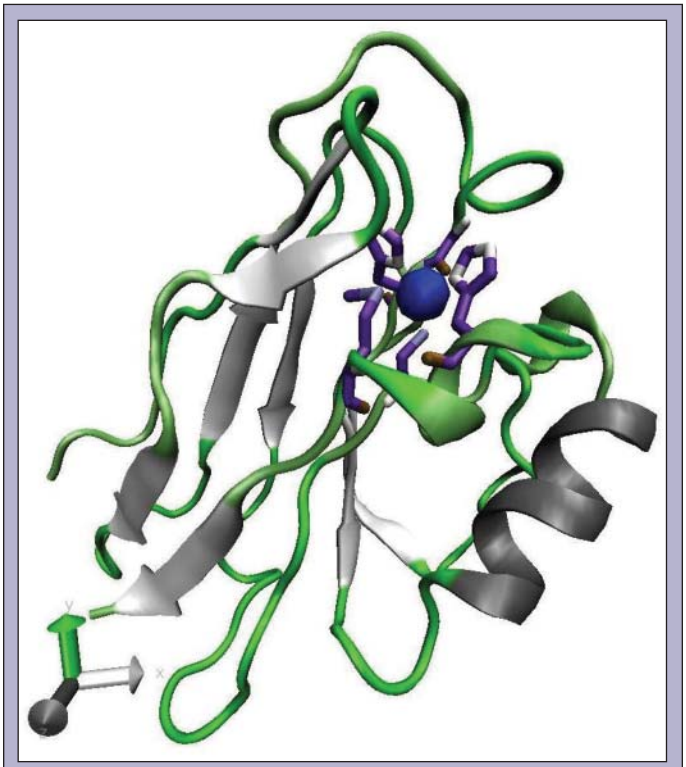

Figure 2. A structural model of the reduced-copper azurin molecule. (Crane et. al., 2001.)

MATERIALS AND METHODS

1. Chemicals and Solutions

Isopropyl-β-D-thiogalactopyranoside (IPTG), Antifoam 204 and Terrific Broth (TB) from Sigma-Aldrich, Rainbow Markers from Amersham (Buckinghamshire, United Kingdom), *E.coli* strain BL21(DE3) from Novagen (Merck KGaA, Darmstadt, Germany) were used . Buffer A was composed of 20% sucrose, 0.3 M Tris, 1 mM EDTA, pH 8. Buffer B was 1 mM $MgCl_2$. Solution C was 0.5 M ammonium acetate (pH 4.1). Solution D was 0.05 M ammonium acetate. All solutions were prepared at 20°C. Cobalt substituted recombinant human carbonic anhydrase II was obtained from Robert Heck (PNNL) and prepared as described in Lipton *et al.* Briefly, the carbonic anhydrase was purified from cobalt supplemented and zinc depleted minimal media cultures of a pET/*E.coli* expression system [5].

2. Cloning and expression system construction

A synthetic gene was constructed by GenScript Corporation (Piscataway, NJ) from the amino acid sequence of *P. aeruginosa* azurin including the N-term targeting sequence. The codon frequency was optimized for expression in *E. coli*, and the gene was cloned into Genscript's pET expression plasmid, pGS-21A, at the NdeI and HindIII restriction sites to give plasmid pGS-azurin. Plasmid DNA was transformed into BL21 (DE3) competent cells.

3. Fermentation and protein overexpression

E. coli transformants containing pGS-azurin were initially grown on LB/Agar (100 mg/L ampicillin) plates and then used to inoculate 50 mL Terrific Broth (TB) shaken cultures (with 100 mg/L ampicillin) at 37°C overnight. 200 mL of overnight culture was used to inoculate a 2 liter New Brunswick Scientific (Edison, NJ) Bio Flow 110 Fermentor filled with 1.6 L TB (100 mg/L ampicillin). Pumps for antifoam, 5 M ammonium hydroxide, and 40% w/v glucose were attached to the system. The temperature, pH, glucose level, and dissolved oxygen level were monitored through the program BioCommand Plus. Temperature was kept at 35°C and pH at 7. Glucose was delivered to the system as necessary, determined through the effect of glucose pulses on the dO_2 level similar to the methods of Akesson [6]. Glucose feed rates increased from 0 to 0.46 mL/min during the six hours prior to induction. A dO_2 level of 30% was maintained through adjustment of first the agitation and, subsequently, the oxygen gas distributed to the system. Cell growth was presumed if motor agitation continually increased. When growth appeared to be stunted at 2.5 hours and without glucose being rate limiting, 10 mL of a mineral solution (0.5 M ammonium sulfate, 1 M KH_2PO_4, 1 M Na_2HPO_4) were added. Cell growth was monitored through the performance of an optical density (OD) measurement every hour after start and before induction.

Induction was performed at 6 hours, when the optical density of the culture was approximately 20, by the addition of 0.1 mM IPTG. Following induction, the glucose feed was reduced to 0.17 mL/min

and temperature was reduced to 25°C. Cells were harvested 17 hours after induction, and used immediately for isolation of protein.

4. Azurin purification

The leader sequence on the azurin protein causes *E. coli* to export the protein to the periplasmic space of the cell [7]. By performing an endosmotic shock procedure on the cells, the azurin can be extracted in a lightly contaminated form, and further purified.

The fermentor cell culture was centrifuged at 3,000 x G for 30 minutes at 4°C to harvest the cells. Wet weight of cells was about 200 grams. The cell pellets were washed twice with 300 mL of buffer A and centrifuged at 6,250 x G for 25 and 30 minutes at 4°C. Because a distinct cell pellet was not formed after the second wash, the cell suspension was centrifuged an additional 20 minutes at 7,000 x G (4°C). The supernatant was decanted once again, and cells were resuspended in 300 mL of buffer B, placed into a -20°C chamber for 10 minutes, then at 4°C for an hour to release periplasmic protein by osmotic shock. This mixture was then centrifuged at 17,000 x G for 20 minutes (4°C) and the supernatant containing azurin was collected. Solution C was added in an amount equal to 1/10 the total volume of supernatant to precipitate out unwanted proteins and the resulting mixture was allowed to settle at 4°C for 24 hours.

At this point two tests were performed to detect the presence of azurin. First, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was executed using a Mini Protean II system (Bio-Rad, Hercules, CA) with 10–20% tris-tricine polyacrylamide gels, at a constant current of 40 amps for approximately 1 hour. Samples were run against Amersham Rainbow Markers. Secondly, a $CuCl_2$ addition test was performed as described below. The SDS-PAGE revealed a dark band at the expected 14 kDa for the mature form of azurin lacking the periplasmic targeting sequence.

The $CuCl_2$ test consisted of adding 0.08 mL of a 0.2 M $CuCl_2$ solution to 30 mL periplasmic extract and observing the ensuing color change. The extract slowly darkened to a deep blue color over the course of 24 hours, indicating azurin presence. Analysis of the sample with a Beckman Model 640 DU spectrophotometer allowed us to form preliminary estimates as to the size of the sample. Due to the size of the sample, it was now split into two equal parts. Half was frozen and stored at -20°C, while we continued our experiments with the other half.

For the solid state NMR experiments, it was necessary to add highly enriched ^{65}Cu to the azurin. 20 mg of 97% isotopically enriched ^{65}Cu Oxide (Isotec, Sigma-Aldrich, St. Louis, MO) was dissolved with 0.6 mL of 12 M hydrochloric acid, dried under a stream of N_2 gas, then reconstituted in water for a 0.1 M concentration. All of the 0.1 M $CuCl_2$ solution was added to the azurin sample, and allowed to sit overnight.

The extract was further purified by high performance liquid chromatography (HPLC) through a 10 x 1.6 cm column with Poros 20 CM weak cation exchange resin (Boehringer Mannheim, Mannheim, Germany). A gradient from pH 4.1 to 5.1 in solution D was used to elute the sample with the major azurin peak eluting at pH 4.75. Fractions exhibiting high absorbance at 625 nm were pooled. The pH was adjusted to 8 with 1.5 M ammonium hydroxide. The sample was concentrated with an Amicon ultrafiltration unit (76 mm 1,000 nominal molecular weight limit (NMWL) membrane, filter code: YM1), dialyzed against 4 L of water overnight, then freeze-dried. A small amount of freeze dried ^{65}Cu azurin was overloaded onto SDS-PAGE to estimate purity (Figure 3). A few milligrams of freeze dried azurin were accurately weighed out, resuspended in dH_2O, and absorbance was measured at 625 nm to determine an accurate extinction coefficient.

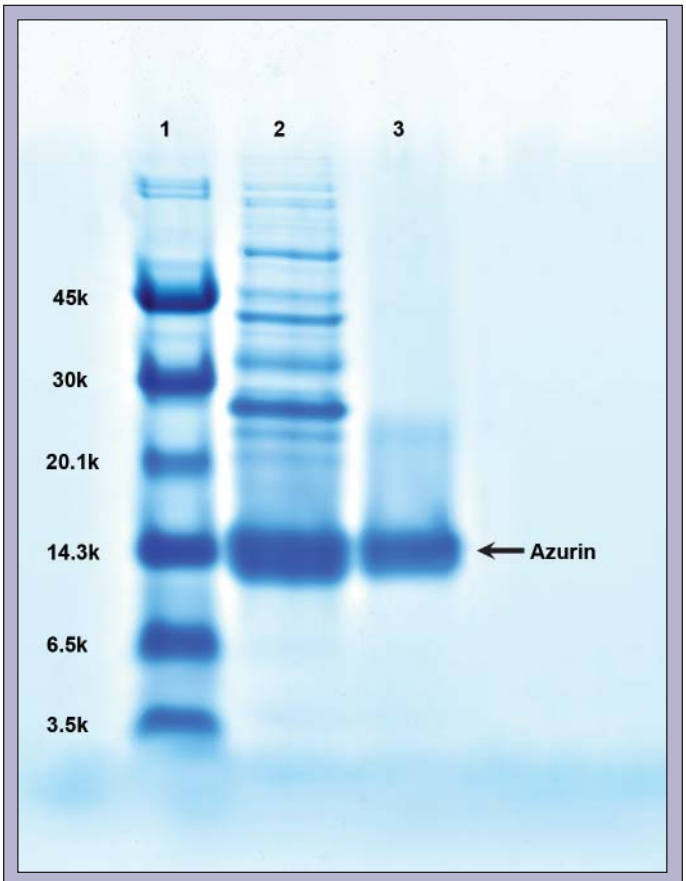

Figure 3. SDS-PAGE gel that confirms the purity of the final azurin sample. Lane 1 contains Low-Range RainbowTM Molecular Weight Markers from Amersham, while lane 2 contains the periplasmic extract from the grown *E. coli* cells, and lane 3 contains a purified azurin sample. Both samples were overloaded onto the gel to identify any contaminating bands.

5. NMR Analysis

70 mg of azurin were weighed out and mixed with 20 mg of cobalt carbonic anhydrase as a dopant [8] in 150 µL of 50 mM ascorbate and 30% glycerol. The ascorbate solution, which is used to reduce the copper in the azurin sample, had been previously sparged with N_2 gas for 20 minutes to eliminate any dissolved oxygen. The final sample of approximately 0.2 mL faded from a dark blue to a clear light purple solution, which was pipetted into a 5x20 mm glass NMR tube for analysis.

A Varian ^{UNITY}*INOVA* spectrometer with a medium-bore (63 mm) Oxford Instruments magnet and an Oxford Instruments

continuous flow cryostat was used to collect NMR data at 18.8 T (227.144 MHz for ^{65}Cu and 799.691 MHz for ^{1}H) and a sample temperature of 10 K. Zinc RF coils were built for this experiment to eliminate interference from the previous copper coils in the probe described in Lipton *et al.* [9]. Solid-state nuclear magnetic resonance was performed as previously described [5, 10] with the following parameters: The proton $\pi/2$ pulse width used for cross polarization (CP) was 7 μs with a 30 ms contact time and a 30 s recycle delay. The ^{65}Cu π pulses used in the quadrupole Carr-Purcell-Meiboom-Gill (QCPMG) train were 19 μs with a τ of 150 μs with a decoupling field of 44 kHz for 512 transients. The ^{65}Cu RF frequency was offset for each experiment to trace the overall pattern of the lineshape.

Results

Azurin production was maximized by fermentation to a high cell density with glucose limited growth and oxygen supplementation and a lengthened post-induction incubation at a reduced temperature (25°C). The average growth rate was 3.3 AU·h^{-1} and the culture was allowed to grow to a density of approximately 10.4 g dry cell weight/L (dcw/L) before induction.

The overnight post-induction incubation, as opposed to a 3 hour post-induction incubation, was shown to result in a higher azurin yield, as well as a lower retention of azurin within the cell membrane where it would be more difficult to harvest.

In the harvest procedure, the wash and endosmotic shock procedures have been streamlined. Centrifuging the cell tubes for 20–30 minutes at 6,250 x G was found to be ineffective in forming a distinct cell pellet a majority of the time. For future methods, it is recommended to centrifuge the tubes at 7,000 x G for a minimum of 20 minutes per step.

Azurin has been previously shown in our lab [11] to be mostly apoprotein following the endosmotic shock procedure. This *apo*-azurin readily takes up Cu(II) ions, resulting in a strong absorbance maximum appearing at 625 nm with an extinction coefficient of 8400 M^{-1} cm^{-1}. Thus, a preliminary estimate as to the total amount of azurin produced was determined at this point by measuring the absorbance of the dark blue azurin sample resulting from the $CuCl_2$ addition with a Beckman DU 640 spectrophotometer. Absorbance was measured to be 2.10 AU at 625 nm, which amounts to between 1,600 and 2,000 mg of azurin, by the estimated extinction coefficient.

Regarding purification of the azurin sample through HPLC, it was noted that the column equilibration time is unacceptably lengthy. This is likely due to the high buffering capacity of the column in conjunction with inadequate buffer strength of the chosen buffers. Therefore, additional experiments must be conducted to determine an appropriate buffer or a more suitable column.

Ultimately, 1.20 grams of ^{65}Cu labeled azurin were obtained. Figure 3 shows the results of SDS-PAGE of the purified protein. By overloading the purified azurin sample, several contaminating bands become visible, but they are estimated to be less than 5% of the total protein present in the azurin preparation. This amounts to approximately 1,500 mg of azurin per liter of culture, and approximately 11.8 mg of azurin per gram of wet cells. In addition, an extinction coefficient of 5,240 M^{-1} cm^{-1} was obtained for azurin.

The composite spectrum or skyline projection of the resulting frequency offset NMR experiments is depicted in Figure 4. The lineshape spans from +1.33 MHz to -2.11 MHz relative to CuCl at 0 Hz (0 ppm). Assuming a quadrupole dominated lineshape, extracted parameters are C_q of 71.2 MHz, η_q of 0.2 and an isotropic shift of 50 kHz from CuCl. The calculated NMR lineshape from these parameters is shown in Figure 4 above the experimental spectrum. The region of intensity at the center of the experimental spectrum is an artifact or impurity and not due to the azurin. This was confirmed by a series of Bloch decay experiments directly observing the copper and the region was found to be more consistent with the simulated spectrum (data not shown).

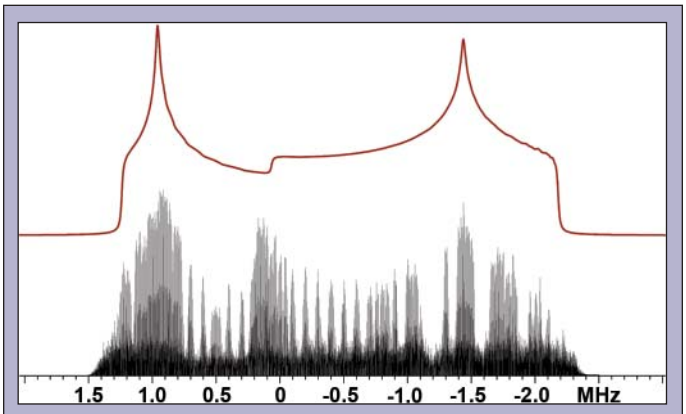

Figure 4. The skyline projection of the various frequency offsets of the ^{65}Cu NMR spectra of azurin at 18.8 T and 10 K with a simulated lineshape in red above it calculated from the parameters listed in the text.

Discussion

1. Optimization of Azurin Production

In past years, much has been done regarding the field of azurin research. Karlsson *et al.* have found a way to easily produce it outside of its host organism [3]. His methods have been improved by Harris *et al.* [2], and the structure of azurin has been defined through x-ray diffraction techniques reported by Crane *et al.* [4]. Our results show that azurin can be produced in higher quantities than before through fermentation.

While manual control of fermentation succeeded in growing *E. coli* to a high cell density, this procedure could be optimized even further. Clearly, there is a limit on the density to which the cells can grow, but there are several examples in the literature of efficient recombinant protein production in *E. coli* to cell densities over twice the level we achieved [12]. The six hour growth before induction could be shortened through a more highly automated fermentation procedure. In the methods described above, antifoam, ammonium hydroxide, temperature, and agitation/oxygen level were automated through proportional-integral-derivative (PID) control, but the glucose feed had to be controlled manually due to limitations in the software and hardware used. The manual control of the glucose feed required a human presence during a majority of

the fermentation procedure. Automation would not be exceedingly challenging. It may be possible for a program to be written within the interface of the BioCommand software, developed separately, or a different system used so that *E. coli* growth would not merely be more efficient, but not require constant supervision. Such a program might give the system a 5–10% glucose pulse every 10 minutes, and adjust the glucose feed according to the methods described by Akesson. That is to say, when the *E. coli* are underfed, the glucose pulse will result in a growth surge and resultant drop in dO_2, while if they are overfed, the pulse will not cause any reaction. This is due to the theory that specific oxygen uptake reaches a maximum at the onset of acetate formation [6]. Acetate formation is crippling to the system, as it has been shown to both inhibit growth and recombinant protein production, possibly even causing the latter to be halted entirely at acetate concentrations between 33 and 167 mM [12]. Therefore, the program might offer the condition that IF the dO_2 level drops below 25%, then the glucose feed will be increased by 2%. Following glucose pulses, a plunge in dO_2, representing normal cellular respiration, would then result in increased feed, while slight or no change in dO_2, representing onset of acetate formation, would not. Such a feedback mechanism would ensure that a glucose level that sanctioned acetate formation would never be reached. The BioCommand program has proved difficult to manipulate to perform this, but it would not be a formidable program to write in a standard programming language, such as Matlab or Visual Basic. One other concern must be taken into account. The Bio Flow 110 is particularly prone to dO_2 oscillations, especially when the glucose level is unusually high. A program to regulate glucose level, then, must offer a safeguard against automatic increases in glucose feed during dO_2 oscillations, such as an alarm or termination in glucose feed.

Due to the blue-green color of the pelleted cells following periplasmic protein extraction during previous preparations, we believed that a fair amount of azurin was not exported to the periplasm of the cell following induction. By reducing the post-induction temperature and not adding Cu(II) until later in the purification we achieved a large increase in azurin which could be recovered from the periplasmic extract. Further optimization of the yield might be achieved by further increasing the cell density at which induction is initiated.

2. Interpretation of NMR results

Sensitivity is a substantial concern in all NMR experiments. A balance must be achieved between high and low sensitivity, for high sensitivity may have high signal, albeit accompanied with high noise. In previous experiments, low signal-to-noise resulted in an inability to detect the copper ion within azurin at all. To circumvent the sensitivity issue, we have made several alterations to our experimental protocol. First, a large amount (70 mg) of azurin was used. A new probe coil, composed of zinc rather than copper (which interferes with the azurin copper signal) was made. To further reduce the possible background signal the magnetization was generated utilizing cross polarization with spin temperature alternation. The sample was brought down to a temperature of 10 K, which increases the Boltzmann distribution of observable spins. While all of these alterations increase the signal-to-noise ratio, the last can potentially increase the T_1 relaxation time to hours. To counteract this, cobalt-substituted carbonic anhydrase is added to the sample as a dopant. The dopant significantly reduces the T_1 relaxation time of the proton bath to the order of seconds or minutes. Any nucleus in the vicinity of the paramagnetic cobalt senses the unpaired electrons within it, which provide efficient nuclear-relaxation pathways [13].

Furthermore, to increase signal, a spikelet echo technique (or QCPMG) is used to continually refocus the signal with a train of pulses. Each complete echo is recorded and a Fourier transform on them produces a series of discrete spikes, whose envelope reveals information about the specific binding site for copper. The timing was set such that the spikes have a 5 kHz spacing between them. A finer distribution leads to more spikes, but a lower signal-to-noise ratio.

The azurin spectrum with its C_q of 71.2 MHz generated a lineshape that is over 3 MHz wide. With the proton $\pi/2$ at 7 μs for CP and the ^{65}Cu π pulse at 19 μs (limited by the voltage handling of the components) the observable bandwidth for each experiment was only 20 kHz. To acquire a complete data set, filling in the lineshape completely would have been time prohibitive. Each frequency offset was acquired with 512 transients, pulsing every 30 s for a total time for each block of 4.25 hours. The spectrum shown in Figure 4 is the result of approximately 3.5 weeks of instrument time.

3. Future directions

While we have obtained an NMR spectrum for the copper (I) in azurin, molecular and quantum mechanical modeling of the site must be performed and the results reconciled with our data. This will tell us whether the proposed models are reasonable and possibly elaborate on key points of the model.

This work demonstrated the feasibility of studying copper in many other metalloproteins by solid state NMR. The trigonal bipyrimidal ligand geometry and axial oxygen ligand of Cu(I) within azurin caused extreme distortions that made it one of the hardest proteins to study with NMR. The extremely large field gradient and the broad NMR spectrum that was generated by it is presumably as large as is likely to be generated by any copper protein, giving promise to the future study of other proteins. The spectra that could be obtained from several other intriguing copper proteins such as superoxide dismutase (21.65 MHz C_q from NQR [14]) should be far simpler to obtain and analyze. Further development on the NMR probe could also lead to a reduction of experiment time making these types of experiments less demanding and more generally applicable to other systems.

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