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Purification and Characterization of Nickel Uptake Regulator (NUR) and Single NUR Mutants from *Streptomyces coelicolor*

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Purification and Characterization of Nickel Uptake Regulator (NUR) and Single NUR Mutants from *Streptomyces coelicolor*

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ABSTRACT

The Nickel Uptake Regulator (NUR) is a metalloregulatory protein found in the microorganism *Streptomyces coelicolor*. *S.coelicolor* is a gram-positive bacterium that plays an important role in antibiotic production. NUR is responsible for the uptake of nickel and the regulation of the enzyme Superoxide Dismutase (SOD) within *S.coelicolor*. NUR regulates Fe-and Ni-SODs through direct and indirect transcription processes, respectively. The goal of this research is to purify and characterize the metal and DNA binding affinities in Wild Type (WT) NUR. There are two metal binding sites within NUR that is believed to contribute to the function of this protein. These sites are the M-site and the Ni-site; both sites are highlighted and enlarged in Figure 1. Our focus in characterizing this protein is to understand the distinct roles each specific metal binding site plays in NUR's functionality. Using optimized purification strategies, we are able to successfully purify protein that is characterized by quantification of original metal content bound to NUR, metal binding affinity, and DNA binding affinity. One experiment, represented in Figure 2 below, shows a titration of NUR and DNA into a solution of a known metal chelator, Fura-2. This experiment presents evidence of our hypothesis that one M-site has a lesser affinity than the other for metal, and in order to remove the other metal, DNA must be bound to NUR. Other experiments will be performed to reproduce this data and give more information to help support this hypothesis.

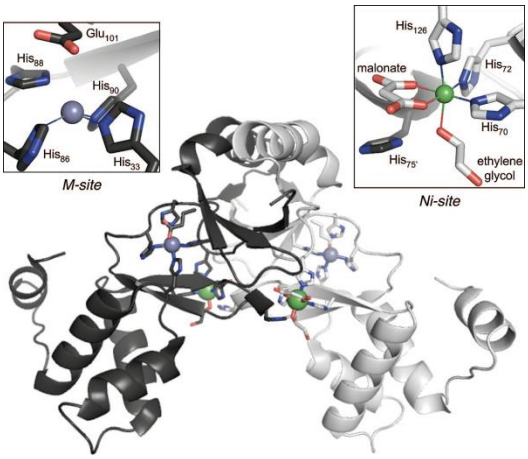

Figure 1. Structure of Nickel Uptake Regulator

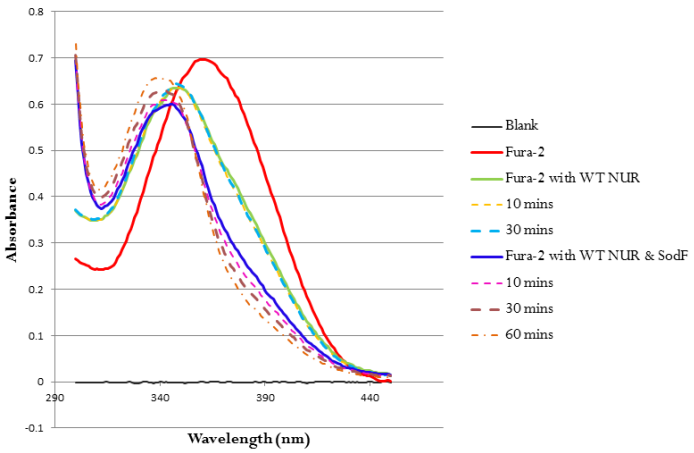

Figure 2. Titration of NUR and DNA into Fura-2

INTRODUCTION

Transitional metals have been found to be used in many biological systems. Ni, Zn, Cu, Co, and Irr are transition metals known to be essential for biological functions. These metals can be found within all compartments of cells and function in multiple processes. In various systems, transitional metals are used in different processes such as electron transfer, redox catalysis and transcription regulation[1]. Specific proteins have an affinity for specific metal ions; the complex formed by the protein and metal is

directed by the selectivity of the protein for the specific metal.[1] For example, the Ferric Uptake Regulator (FUR) binds intracellular iron to maintain adequate iron levels in order to avoid toxic levels within the cell. FUR also regulates transcription of certain genes within the various organisms it is found in. The presence or the absence of transition metals influences the cellular process that each metalloregulatory protein is responsible for. It is important to understand how proteins use the metals in their environment so that the functional and structural properties of these proteins may be better understood. Furthermore, when the optimal metallic conditions for such proteins are known, it allows for a deeper understanding of how these proteins behave in the different cellular systems.

The use of transition metals in cells is vital to maintaining cell life. Too high or too low concentrations of metal within a cell can lead to cellular stress. This stress can disrupt the cell's function. This disruption can interfere with its ability compensate for the absence of metal or the ability to alleviate excess metal within the environment.[1] The ability to maintain adequate concentrations of metal ions is referred to as metal homeostasis.[1] This ability is necessary for cells to function properly. Metal homeostasis is maintained by transportation, efflux or storage metal ions via specific metallochaperones. Metal homeostasis is essential for cell function; therefore certain proteins are solely responsible for this purpose. Trafficking, efflux, and storage of metal within a cell are controlled by metal sensing proteins. The way metal ions move and where they move to is reflected by their effect on the cell. Metallochaperones are proteins responsible for transporting metals throughout the cell. Transcriptional regulator proteins use metal to regulate the expression of genes.[3]

To maintain homeostasis, it is the job of metalloregulatory proteins to see that the efflux, storage, and uptake of metal ions are performed. The proteins, when their respective metal ion is bound, will either repress or activate transcription of the gene whose promoter region is bound by the protein.[3] This new complex of protein-metal-promoter can activate transcription of a gene and/or repress the transcription of a gene. The specific transcription regulatory protein we are focusing on in this study is the nickel uptake regulator (NUR).

METHODS

Production of Media

Escherichia coli (E. coli) were grown in Lauria bertani broth (LB). This media was made by adding 20g of pre-made LB stock to 1 liter of deionized water.

Inoculation of Cells

This process was completed under sterile conditions. In 250 mL autoclaved L.B., 250μL of ampicillin was added. A micropipette was used to transfer ~10 μL of E.coli NiCo (DE3) cells harboring a pET3a plasmid with the NUR gene inserted between the Nde1 and BamH1 sites, into the flask. This solution incubated overnight at a temperature of 37ºC.

Expression of Cells

The overnight culture was equally distributed in volumes of ~40mL into each 1L flask of L.B. To each flask 0.05 mg/mL ampicillin was added. The flasks were shaken at 160 rpms and 37ºC until optical density$_{600nm}$ of 0.6 was reached. Cell density was checked using the Hewlett Packard DU 800 Spectrophotometer. Once O.D. was reached, protein expression was induced with 400μL Isopropyl β-D-1-thiogalactopyranoside (IPTG). Once induced, the cells incubated at 37°C for 4 more hours. The cells were harvested by centrifugation. The cell pellet was collected and stored at -20°C.

Cell Lysis

The harvested cells were re-suspended in Buffer A (25mM Tris, 100mM NaCl, 1mM Sodium Dithionite at pH 8). Sodium Dithionite was added to Buffer A after it was degased. Cells were re-suspended with ~75mL of Buffer A. To the re-suspended cells, a 1:1000 dilution of 100mM Phenylmethylsulfonyl-Fluoride (PMSF), and DNase were added to the re-suspended cells for lysis. The cells were lysed on ice by sonication.

Protein Purification

The optimized purification strategy involves Immobilized Metal Affinity Chromatography. Buffer A consists of 25mM Tris, 100mM NaCl, 1mM Sodium Dithionite at pH 8. Buffer B consists of 25mM Tris, 500mM NaCl, 1mM Sodium Dithionite, and 500mM Imidazole at pH 8 and was used for the gradient. The lysate loaded on the IMAC column had a concentration of 1M NaCl. The conductivity lowers before the Buffer B gradient that elutes off pure NUR begins.

Protein purity of each fraction was determined by 15% SDS-polyacrylamide gel electrophoresis. Fractions containing NUR were collected and precipitated with 30% ammonium sulfate.

Gel filtration of cellular proteins is the next step in purification of NUR protein. A 25mM Tris, 500mM NaCl buffer at pH 8 containing 1mM DTT (Dithiothreitol) was used as the elution buffer for this step. The protein sample was injected into the GF75 column, which separates proteins according to size. After each injection, 1mL fractions were collected of the peaks shown by the chromatogram. After this step, fractions where we anticipated NUR to be were chosen to run by SDS-polyacrylamide gel electrophoresis.

Quantifying Protein Concentration

Fisher NanoDrop spectrophotometer was used to quantify the concentration of protein by monitoring the absorbance at 280nm using the ε_{280}=10000 $M^{-1}cm^{-1}$. From here, we were able to design metal binding assays.

Metal Quantification

The Atomic Absorbance spectrophotometer is used to determine the % metal occupancy in purified NUR. NUR is tested against zinc and nickel lamps. Standards for Zn are made from 0.25-1ppm in increments of 0.25ppm. Ni standards are made from 1-4ppm in increments of 1 ppm. The instrument is standardized in every experiment before testing NUR. Data is analyzed to determine the %Zn/Ni occupied within NUR protein.

Metal Binding Assay

Nickel was titrated into the flurofore, MagFura2, to determine the actual concentration of MagFura2 at 10μM. This experiment was repeated three times for validity.

We conducted our metal binding assay for each NUR protein the same way. A solution of 20μM NUR and 10μM MagFura2 diluted to 1mL with Tris buffer was prepared in a cuvette. 400μM Ni^{2+} is titrated into the cuvette up to 1% volume and analyzed via Fischer NanoDrop spectrophotometer; the data was exported into excel to be analyzed and then the data was fit with the program DynaFit4.

RESULTS

Purification

Escherichia coli BL21 NiCo cells are grown and induced with IPTG to express NUR. After lysis, the lysate plus 1 M NaCl is run through the nickel loaded metal chelating affinity column (MCAC). Figure 1 is a 15% SDS-Polyacrylamide gel electrophoresis image that shows samples collected after IMAC purification. A band with estimated purity of > 90% of protein at approximately 16kDa is shown in this image. The dark band at this molecular weight indicates that we have collected protein with a molecular weight that is close in proximity to that of NUR's actual molecular weight, 16229 kDa. The next step in purification is gel filtration chromatography. Figure 2 is an SDS-Polyacrylamide gel electrophoresis image that shows a band with an estimated purity > 95% at ~ 16kDa.

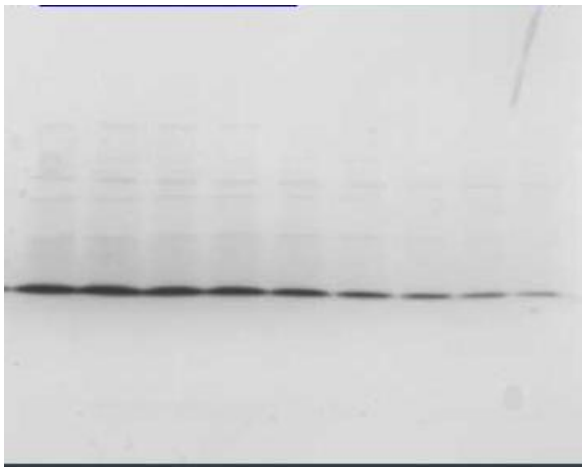

Figure 1. SDS-PAGE image of MCAC chromatography of WT NUR.

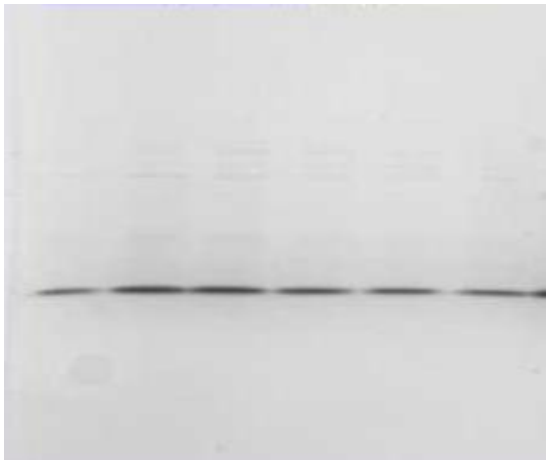

Figure 2. SDS-PAGE image of Gel Filtration chromatography of WT NUR.

Metal Quantification

After purification is completed, the concentration of protein is determined using the Fischer NanoDrop spectrophotometer. Absorbance is measured at 280 nm with a molar absorptivity of 10,000 $M^{-1}cm^{-1}$; this data is then used to calculate concentration. A 10:1000 dilution of protein and buffer is made to measure the concentration. Wild type NUR has a concentration of 233μM. Next, the original metal content is quantified via the atomic absorbance spectrophotometer. NUR is tested for nickel content and zinc content. Nickel standards are made at 1, 2, 3, and 4 ppm and zinc standards are made at 0.25, 0.5, 0.75, and 1 ppm. Every purified NUR protein is compared to these standards. Based on Figure 3, Wild Type NUR was found to have an average absorbance of 0.004 from the Ni standards. From these data, it is estimated that NUR is purified with ~0.4 Ni ions per monomer.

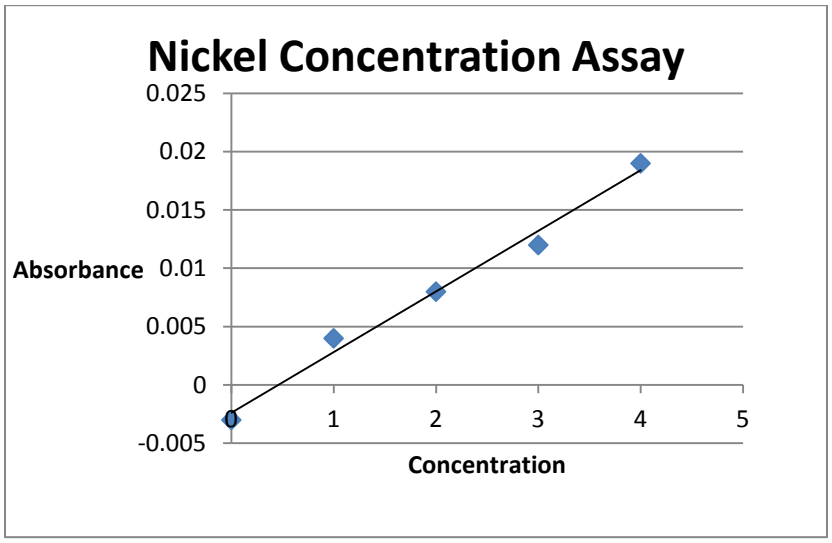

Figure 3. The standards made were at concentrations of 1,2,3 and 4 ppm. The linear regression equation for the nickel standards is y = 0.0052x - 0.0024 with an R^2 value of 0.9869.

When testing NUR with the zinc standards, the average absorbance of Wild Type NUR was 0.0313. This absorbance correlates to approximately 0.23 ppm Zn. The nickel and zinc standard data suggest that NUR is purified with roughly 1 metal ion per monomer (~0.4 Ni and 0.6 Zn).

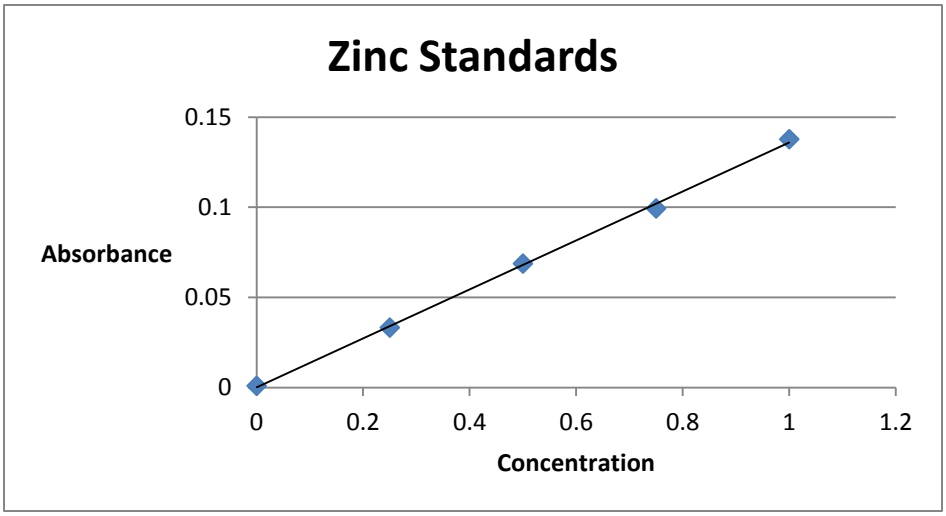

Figure 4. Zinc standards were made at concentrations of 0.25, 0.50, 0.75 and 1.00 ppm. The linear regression equation for the standard curve is $y = 0.1359x + 0.0001$ with an R^2 value of 0.999.

DTNB ASSAY

After calculating the original metal occupancy, a DTNB (Dithionitrobenzoate) assay is completed to determine the amount of free thiols present in NUR. NUR contains 4 cysteine residues per monomer, so a maximum of 4 free thiols may be determined. For this experiment, 4 solutions of varying amounts of protein, 50µL DTNB, all diluted to the same volume with buffer (25mM Tris, 100mM NaCl, ph8) are tested using the Fischer NanoDrop spectrophotometer. Absorbance is measured and compared to the concentration of protein to resolve the amount of free thiols present. We expect to find 2 free thiols based on the crystal structure that indicates that 2 of the cysteine residues are present as a disulfide, leaving only 2 cysteines predicted to be reactive. In Wild type NUR, it was calculated that there are 2 free thiols present, as expected.

DISCUSSION

The Nickel Uptake Regulator found in *Streptomyces coelicolor* has been found to regulate levels of nickel within the cell. Lysis at high salt and purification at low salt via Ni-MCAC and gel filtration chromatography resulted in pure Wild Type NUR (estimated > 95%). The image of a single dark band at 16kDa is confidence that it is majority NUR in our fraction samples.

In the structure of NUR, it has been found that within the m-site, Zn is bound and in the ni-site, nickel is found. From the atomic absorbance spectrophotometer quantification, Wild type NUR was found to be 60% Zn loaded and 40% Ni loaded, which is consistent with past research. In the past it was found that Wild Type NUR contained 1 metal ion per monomer and current data verifies this. The DTNB assay proved that in Wild type NUR, there is a disulfide bond which leaves two reactive thiols. As preliminary metal binding experiments are concluded and the data is fit, the binding constant for Ni into NUR will be determined.

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