STABILIZING THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) BY NUCLEOTIDE DERIVATIVE BINDING TO PROMOTE PROPER FOLDING

A Senior Honors Thesis

by

RYAN CRAIG SMITH

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2001

Group: Biochemistry

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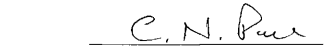
Approved as to style and content by:



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April 2001

Group: Biochemistry

ABSTRACT

Stabilizing the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by Nucleotide Derivative Binding to Promote Proper Folding. (April 2001)

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Seventy percent of people who suffer from cystic fibrosis have a cystic fibrosis transmembrane conductance regulator gene on chromosome 7 that contains a three base-pair deletion of phenylalanine at position 508, in a nucleotide binding domain called NBD1. Nucleotide binding domain one (wild-type) was expressed in protein aggregates, or inclusion bodies, from a plasmid in *Escherichia coli*. It was then purified on a nickel histidine-binding column and refolded. A fluorescence emission spectra wavelength scan was run on the protein that yielded a λ_{max} of 330nm for the native state of NBD1 and a λ_{max} of 346nm for the denatured state. The biggest difference between the two curves was at 225nm so this wavelength was chosen to follow unfolding. Monitoring the denaturation of NBD1 with fluorescence at this wavelength will give a characteristic curve that can be used for comparison with binding ligands to the mutant phenylalanine 508 deficient form in order to find any stabilizing effects that promote proper folding.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Pace, for accepting me into his laboratory without prior experience. Thank you for taking a gamble by sponsoring me as a research fellow. I would like to thank my fellow lab workers, Stephanie, Saul, and Lee especially, for their invaluable assistance in the work of this project and the rest of the Pace lab for their willingness to put other projects aside temporarily to answer my questions. I would also like to thank my family for their continual support.

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INTRODUCTION

Cystic Fibrosis is a disease that displays recessive Mendelian heritability and causes the clogging of passageways in organs with thick mucous secretions. It is given its name from the microscopic observations of the symptoms it causes in the pancreas, which look like cystic fibers. The biochemical function of the protein responsible for causing these symptoms is a chloride ion transport called the cystic fibrosis transmembrane conductance regulator that has a transmembrane domain, two nucleotide (ATP) binding domains, and a regulatory domain. The diseased, cystic fibrosis causing, form of the protein has the deletion of a phenylalanine at position 508 (FΔ508), which is in the first nucleotide binding domain (NBD1). This phenylalanine deletion is present in 70% of cystic fibrosis cases and disables the protein from folding into its correct conformation labeling it for degradation in the endoplasmic reticulum. Therefore, the FΔ508 mutation form of the protein never makes it out of the endoplasmic reticulum to the golgi apparatus to the cell membrane where its function is carried out, and the ability to transport chloride in and out of the cell is lost.

This thesis follows the style and format of *Protein Science*.

There has been an influx in knowledge as to the function and structure of CFTR in recent years but a means of stabilizing it to promote proper folding in the endoplasmic reticulum has not been explored. It has been shown that binding molecules to proteins can have allosteric effects, causing them to take on a new conformation or stabilizing the previous conformation. The binding of nucleotide derivatives such as AMP-PNP (Weinreich et al., 1999), which cannot be hydrolyzed, to NBD1 has been studied in the sense of discovering the transport mechanism of CFTR, but not to measure any stabilizing effects they may induce. We believe that binding of ligands to NBD1 may have stabilizing effects that could promote correct folding in the endoplasmic reticulum and cause CFTR to make it to the cell membrane to perform its function.

The preliminary investigations that must occur prior to doing the key experiments required for this research involve characterizing the properties of the functional wild-type form of NBD1 of CFTR. Fluorescence was chosen for this characterization because of the presence of a tryptophan residue in NBD1's amino acid sequence and because only a small amount of protein will be required for the experiments.

MATERIALS AND METHODS

Materials

Buffers purchased from Novagen for column chromatography include strip buffer (20mM Tris-HCl, pH 7.9, 100mM EDTA, 0.5M NaCl), wash buffer (30 mM imidazole, 20 mM Tris-HCl, pH 7.9, 0.5M NaCl, 6M GndHCl), charge buffer (50 mM $NiSO_4$), elution buffer (0.4M imidazole, 20 mM Tris-HCl, pH 7.9, 6M GndHCl), binding buffer (5mM imidazole, 20mM Tris-HCl, pH 7.9), and dialysis buffer (100mM Tris-HCl, pH 7.4, 2mM EDTA). The NBD1 refolding buffer contained: 0.385M L-Arg, 0.1M Tris-HCl (pH 7.6), 2mM EDTA, 200mM GndHCl, 1mM DTT, and 4μM NBD1.

Expression and purification of NBD1

The gene that codes for NBD1 was given to us as a plasmid that directs expression of NBD1 in inclusion bodies in *Escherichia coli* by Dr. Phil Thomas of UT Southwestern Medical School. A clonal colony was plucked from a culture plate that selected against cells without the plasmid with the antibiotic kanamycin. The plasmid gave the transformed cells resistance to the antibiotic. This plucked colony was grown in kanamycin LB broth until it reached an optical density at 600nm of 0.6 according to the

protocol provided us by Dr. Thomas and Novagen. The bacteria were induced to express the plasmid with IPTG and centrifuged to pellet the cells. The cells were sonicated after respuspension in binding buffer (5mM Imidazole and 20 mM Tris-HCl, pH 7.9) and centrifuged again. The inclusion bodies were resuspended in binding buffer plus 6M guanadine hydrochloride, and spun down a final time. The supernatant was then run through a nickel His-binding column acquired from Novagen to separate our protein, NBD1, which was tagged with a series of histidines. The eluted protein precipitated during dialysis and was centrifuged and air-dried after being aliquoted into 1.5 ml Eppendorf tubes.

Refolding

The dialyzed protein was resuspended in 30µl of 6M guanidine hydrochloride and the concentration was determined by UV absorbance using the extinction coefficient of 16170 $M^{-1}cm^{-1}$ for NBD1 (wt). This volume of the solution was used to calculate the amounts of the reagents listed above to be added to the refolding reaction to create the proper molarities.

Fluorescence characterization

A fluorescence emission spectra was run on the native and unfolded states of NBD1 to determine the wavelength where the greatest difference in intensity between their curves is observed (Pace, 1986). NBD1 was denatured in 8M guanadine hydrochloride. The native and denatured forms were excited at 278nm to produce a fluorescence emission spectra.

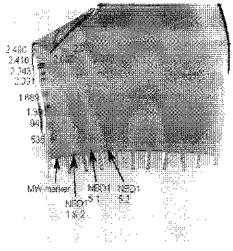
RESULTS

SDS-Page Electrophoresis

The presence and purity of NBD1 was determined by comparing SDS-Page electrophoresis molecular weight markers with the relative band migration distance of our purified protein. The NBD1 1&2 lane in Figure 1 contains protein purified from our first and second trials in November of 2000. The lanes with NBD1 5.1 and 5.2 are from the fifth purification run done from the second expression in February of 2001. The lane labeled 5.1 is from the first half of the purification run. We ran out of elution buffer half way through the run so 5.2 was run in a separate lane to ensure that nothing else came off of the column during this second phase of elution. Figure 1 shows that our protein

band has a molecular weight similar to the expected molecular weight of 30,000 for NBD1. This was determined by comparing the migration distances of the known molecular weight marker bands to those of our protein. The single band in each lane shows that our protein is pure. There was approximately 20mg of protein yielded for each purification.

A



B

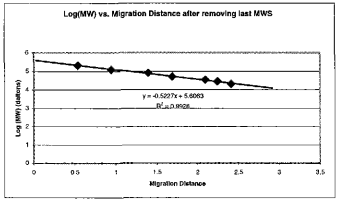


Fig. 1. SDS-Page gel electrophoresis of NBD1 and molecular weight calculations. **A**: Lane one is a molecular weight marker used for comparison with other lanes. NBD1

1&2 is protein from the first and second purification trials. NBD1 5.1 is protein from the fifth purification trial and NBD1 5.2 is the second half of that trial when more elution buffer had to be made. **B**: The last molecular weight standard (MWS) was removed because it deviated drastically from the line shown. The line shows that the molecular weights of our protein in lanes NBD1 1&2, NBD1 5.1, and NBD1 5.2 are 32,648, 32,964, and 32,243 respectively.

Fluorescence emission spectra of NBD1

The wavelength at which NBD1 will be monitored for the unfolding reaction in urea was determined by measuring the fluorescence emission spectra after exciting at 278nm. Figure 2 shows that the optimal wavelength to monitor unfolding is at 225nm, which is the maximum of the difference of the native and denatured curves. The shift in λ_{max} from 346nm for the denatured state to a λ_{max} of 330nm for the native state shows that the tryptophan residue is in more of a nonpolar environment in the native state. This means that it is partially buried in the hydrophobic core of the folded protein and is more exposed to the solution when denatured.

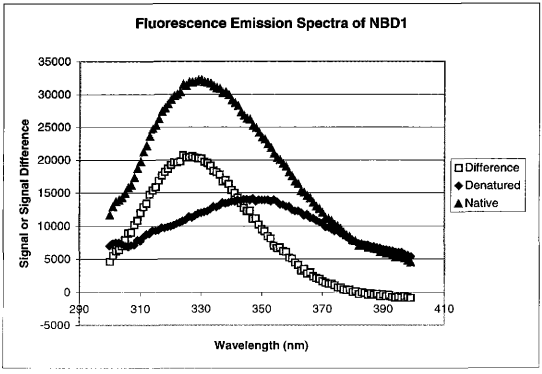


Figure 2. Fluorescence emission spectra with excitation at 278nm of NBD1 in the native and denatured states and the difference between the two curves. The optimal wavelength is the maximum of the difference curve at 225 nm.

DISCUSSION

NBD1 Expression

The main body of accomplishment that was achieved in this research was adding to the knowledge of the proper expression of NBD1. The amount of time that was consumed in the learning curve of a project new to the lab was unforeseen, as it usually is. The present NBD1 expression system in inclusion bodies has proven to be labor intensive and unreliable. The system was originally invented for use in studying the possibilities of refolding NBD1, which resulted in the technique used for this research. It has been proposed by Dr. C. Nick Pace of the Department of Medical Biochemistry and Genetics at the Texas A&M University Health Science Center that an expression system similar to that used for RNase Sa (Hebert, 1997) might produce a higher yield. In this case the protein is expressed in the periplasmic space of *E. coli* and might not require refolding or sonication. This might have definite advantages over the present system if it could be done.

Fluorescence Emission Spectra and Denaturation Curve

The fluorescence emission spectra that we determined agree with the results of Dr. Thomas (Qu, 1996). Likewise, his results showed that the optimal wavelength to monitor the unfolding reaction was 225nm. The only denaturation curve we had time to attempt using this wavelength turned out to be anomalous because we included NBD1 that had been refolded from the first purification and had set since November of 2000. We discovered later from collaboration with the Thomas lab that NBD1 must be refolded again after setting for four hours. Therefore, the data points we obtained were all over the chart and useless.

Conclusion

Although we were not able to realize the goal of monitoring whether or not there are stabilizing effects of ligand binding to NBD1 it is still a worthwhile goal with much potential. A crucial step would be to find a molecule that binds very tightly to NBD1 so that low concentrations would promote the folding of NBD1. Inhibitors of HIV-1 protease have been found with binding constants as low as 0.4 fM (Xie, 2000). If such a molecule could be found for NBD1, experiments could be done using fluorescence to study if there are any stabilizing effects, leading to a possible remedy to the misfolding of CFTR in the endoplasmic reticulum.

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VITA

My name is Ryan Craig Smith and my permanent address is 2306 Ollie St., Corpus Christi, TX 78418. I will receive a B.S. in genetics and graduate with university honors in December of 2001. My work experience includes membership in the Texas Army National Guard as a mortar gunnery operator. I have also worked with TTI as a survey data collector collecting data regarding the safe use of child car seats. Honors that I have received include: General O.R. Simpson Corps Honor Society member, Dean's Honor Roll, Golden Key member, National Society for Collegiate Scholars member, Sigma Xi Scientific Research Society member, General Moore Award (best overall unit in the Corps of Cadets).