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# BARNASE AS A MODEL FOR THE DENATURED STATE OF PROTEINS

A Senior Honors Thesis

by

LEE MICHAEL HOFFART

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 2002

Group: Life Sciences II

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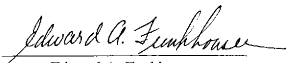
Approved as to style and content by:

C. Nick Pace
(Fellows Advisor)



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April 2002

Group: Life Sciences II

# ABSTRACT

Barnase As A Model For The Denatured State Of Proteins (April 2002)

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Protein folding is one of the major thrusts of biochemical research today. It is the study of how proteins fold into their tertiary structure based solely on their sequence of amino acids. It was once believed that no interactions were made in the denatured state and it does not contribute to stability of the native state. It is now known that this is false. There is structure in the denatured state and it contributes to the stability of the native state. It has become apparent that new techniques are needed to probe the denatured state and learn about its properties. It was proposed to use chemical modification of a cysteine mutant of Barnase to disrupt the hydrophobic core and denature the protein under physiological conditions. Then a series of size exclusion chromatography experiments would then be carried out to characterize the extent to which the protein unfolds. These experiments would be done in increasing osmolyte concentration, which will cause an open conformation to become more compact while an already compact conformation will change very little. Unfortunately, high Barnase yields could not be obtained with the first method of preparation and work was delayed significantly while a new method was investigated. A new expression vector and protocol was

obtained from Bob Hartley at the NIH and implemented successfully two weeks ago.

Work is ongoing now that problems with the expression system have been solved

# ACKNOWLEDGMENTS

I would like to thank Dr. Nick Pace and Dr. Martin Scholtz for their advice and dedication. This work was inspired and supported by them, and without them none of this would have been possible. I would also like to thank Dr. Robert Hartley at the NIH for assisting us with implementing his procedure for Barnase purification. Without him we would still be stuck with the old method of purification. I need to thank David Schell and Saul Trevino for their support and advise on all practical matters of laboratory work as well the rest of the Pace/Sholtz lab for their support.

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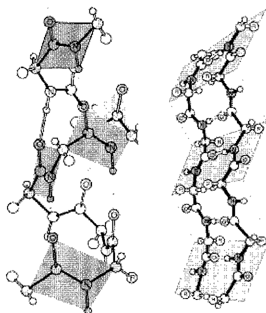
# INTRODUCTION

Protein folding is the study of how and why proteins fold the way they do. Proteins are the building blocks of life, the cellular machines that perform virtually all metabolic functions as well as forming the structures of many living organisms. They are chains of large numbers of 20 different amino acids, whose sequence is determined by an organism's genetic code. This is called the primary structure of the protein. But it is not enough to simply produce a chain of amino acids; these chains must fold into structural elements, called β-sheets and α-helices. These structural elements are referred to as the secondary structure. Figure one shows a typical α-helix and β-sheet, note that non-covalent bonds, specifically hydrogen bonds, hold these structures together. The formation of secondary structure is also not enough to confer function to proteins. It is necessary for these secondary structure elements to fold and group together into what is referred to as the tertiary structure. It is the tertiary structure that provides for the functionality of monomeric proteins. This structure, like secondary structure is held together by non-covalent bonds, with the notable exception of the formation of disulfide bonds between cysteine amino acids. Disulfide chemistry is important for this work in a different way and it will be discussed later. It should be noted that the burial of hydrophobic surface area is important in stabilizing the native state.

The tertiary structure of proteins is determined by the primary structure. The fundamental question of biochemistry is how this happens. How does the linear sequence of amino acids provide the information necessary to fold a protein into its

Note: This article follows the format of Biochemistry for all figures, figure legends and references.



tertiary structure, furthermore how does the protein get from its unfolded state to its folded state?

In the laboratory urea and guanidine-HCl are commonly used as denaturants. A denaturant is a compound that proteins unfold in. Originally it was believed that the unfolded form of the protein was a random coil that rapidly oscillates through time in the presence of denaturants, without any secondary of tertiary structure, much like an organic polymer. It was concluded that the unfolded form of the protein contributed nothing to the thermodynamic stability of the folded form (1, 2, 3). The stability of the folded form is defined as the change in Gibbs' free energy between the folded and unfolded state ($\Delta G$). This proved to be incorrect, and structure has been found in the denatured state in a number of cases (4, 5). In the interim a number of techniques were developed to probe the native state, including the very powerful x-ray crystallography and 2-D nuclear magnetic resonance (2D-NMR). These techniques provide a structure of the folded form of the protein, and from them we can determine the some of the forces that contribute to the stability of the protein. They work in part, due to the fact that under native conditions almost all of the protein populates the native state.

Since, there is some structure present in the denatured state, it is insufficient to determine the forces present in the native state. The forces and structure of the denatured state, in urea or other chemical denaturants, also contribute to the thermodynamic stability of the native state (often times negatively). The detailed structural techniques used to get structures of folded proteins are not useful in getting the limited structure of denatured proteins, because even though there is some structure present, much of the denatured state is unstructured and all of the individual

conformations present at any given time create too much background noise to get a picture of the structured region. There is now a need to develop techniques to probe the denatured state.

Some tools that are used now will be useful in probing the denatured state. Fluorescence spectrophotometery allows for the determination of the environment around tryptophan residues; if they are on the surface, exposed to water, fluorescence intensity is usually high, where as if they are in the core intensity tends to be lower. In general fluorescence provides a way to test for tertiary structure near tryptophan. Since tertiary structure is wiped out for the most part in the denatured state it is possible to follow the folding and unfolding of proteins that contain tryptophan using fluorescence, there are several advanced fluorescence techniques, one of which will be described later.

Circular dichroism (CD) spectrophotometery is based on the effect that α-helical secondary structure has on circularly polarized light, thus it is a good measure of the amount of α-helix present in a protein. As helical structure decreases under denaturing conditions thus, like fluorescence, it may be used to monitor folding and unfolding. For this technique to work there must be sufficient amount of helix present in the native state.

Since the goal of this work is to develop a new technique for probing the denatured state of proteins it is important to choose a protein that is well characterized. Thus Barnase was chosen for this study, since it is one of the best characterized proteins available.

Barnase is an extra-cellular ribonuclease from *Bacillus amyloliquefaciens* with a $\Delta G$ of about 8 kcal $mol^{-1}$. It consists of 110 amino acids, none of which are cysteine, implying that Barnase has no disulfide bonds. It contains three tryptophans, which will allow for fluorescence studies to be performed. There is indirect evidence that Barnase has one of the most extended denatured states in the presence of chemical denaturants like urea (6).

The folding kinetics of Barnase also provides an interesting, and long standing, question. The kinetic studies in question involve unfolded Barnase in 6M guanidine-HCl being diluted into a much larger volume of water and rapidly mixed, reducing the concentration of denaturant. Then refolding is monitored using CD or fluorescence spectrophotometery as described previously. The mixing time is referred to as the dead time of the instrument. In the dead time Barnase undergoes significant change, in other words its signal return when monitoring starts, following the dead time, is different from the signal of denatured Barnase (7). This is referred to as the burst phase; some argue that the burst phase is a stable intermediate with stability as high as 3 $^{kcal}/_{mole}$. Others argue that there is no such stable intermediate, just a burst phase (8).

The goal of this project is to show that chemical modification of a residue in the hydrophobic core can be used to disrupt the folded form of Barnase, and allow the unfolded form to be studied under non-denaturing conditions (in the absence of denaturent). Furthermore it is desired to determine if under these conditions Barnase has as extended a conformation as predicted indirectly. Chemical modification of a protein is easiest achieved at a cysteine residue, thus it is necessary to first make a mutant of Barnase. Fortunately such a mutant had already been made; the alanine at

position 74 was mutated to cysteine. This mutant will be henceforth referred to as Barnase A74C.

## EXPERIMENTAL DESIGN

To successfully perform chemical modification it was necessary to express large quantities of Barnase A74C, and purify it. Before any experiments are done in earnest it is necessary to express between 500 and 700 mg of Barnase A74C. This step proved to be a great stumbling block and was only overcome two weeks prior to this writing. Large quantities of protein are needed because chemical modification is not very efficient in many cases. To do this two purification schemes were used.

*Expression and Purification Scheme I:* Barnase is expressed in a bacterial system. The Barnase and Barnase A74C gene had previously been inserted into a plasmid under control of the lac operon with a phoA leader sequence; this plasmid confers ampicillin resistance to any bacteria that contains it. The phoA sequence causes the protein to be transported to the periplasm, where the leader sequence is cleaved. From the periplasm Barnase may move into the media. The plasmid also contains the gene for Barnase's inhibitor Barstar under its natural promoter, which is constitutively being expressed at low levels. This is necessary to keep leaky expression of Barnase from killing the cells before induction. Sixty μl of calcium chloride competent *E. coli* of the MQ cell line were transformed by placing them for 20 minuets on ice, then heat shocking them at 42°C for 90 seconds, then placing them on ice for 60 seconds. Then 800 μl of LB media is added and they are incubated at 37°C for 40 minuets. Following the incubation 200 μl is plated on agar with 100 $^{\mu g}/_{ml}$ of ampicillin present. The plate is incubated overnight at 37°C, and a colony is picked from this plate to grow overnight at

$37^{\circ}C$ in a 60 ml LB starter culture. Many colonies may be used from the same plate; it is unnecessary to do a new transformation for every preparation of protein.

Six liters of M9 minimal media (42 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 9 mM NaCl, 19 mM $NH_4Cl$, 2 mM $MgSO_4$, 100 µM $CaCl_2$, and 11 mM Glucose), is made with 500 ml measured into each of 12 two-liter flasks. The media is used to grow the *E. coli* in mass. Ampicillin is present at 50 $^{\mu g}/_{ml}$ in the media. They were grown at $37^{\circ}C$, under agitation until the optical density at 600 nm was between 0.6 and 1.0. Then the ampicillin concentration is brought to 100 $^{\mu g}/_{ml}$, since ampicillin degrades at $37^{\circ}C$ in about 8-12 hours and IPTG is added to induce production of Barnase. The temperature is turned down to $28^{\circ}C$ at this point as Barnase is more active at $37^{\circ}C$, and after induction Barstar can no longer inhibit all of the Barnase being produced. It is allowed to incubate for 48 hours, before 25 ml of glacial acetic acid is added to each flask. Ten minuets are the allowed to pass. This step ensures that all of the Barnase that was expressed is now in the media is released from the periplasm.

The media with cells is then loaded into Beckman bottles and centrifuged at 4550 G for 20 minuets. Following this step the supernatant from all of the Beckman bottle is combined into a 6 L flask and the pellet is discarded. Twelve ml SP-trisacryl M cation exchange resin is added to the flask. This stirs at $4^{\circ}C$ for an hour, allowing the Barnase to bind to the resin. Then the resin is loaded onto a column and washed with 50 mM ammonium acetate buffer at pH 4.5, until the flow through, as measured by absorbance at 280 nm reaches a baseline. In this case the measuring of the absorbance is automated, as the solution flows from the column it passes through a spectrophotometer that measures the absorbance. Proteins that do not stick to the resin

are being washed from the column leading to the absorbance. Barnase remains stuck to the resin until 50 mM ammonium acetate, 1 M NaCl buffer at pH 4.5 is added. This screens the electronic interactions keeping Barnase bound, eluting Barnase from the resin. Fractions are collected during the elution step, which are tested on an agar plate containing RNA and the indicatior Toluidine Blue-O at pH 7.0. If Barnase is present it digests the RNA, which makes the environment more acidic thus causing the indicator to change from blue to pink. The fractions that test positive are pooled and dialyzed against 50 mM ammonium acetate to reduce the high salt concentration.

The absorbance is taken of the reaming solution at 280 nm, and the concentration calculated using the extinction coefficient of 27366 $mM^{-1}$ $cm^{-1}$ (9). Multiplying by the total volume determines the approximate yield. An SDS-PAGE gel was then run at with different concentrations of the final solution where impurities that contributed greater than 2%, 1%, 0.67%, 0.33% and 0.11% to the absorbance at 280 nm will show as bands on the gel.

*Expression and Purification Scheme II:* Bob Hartley at the NIH provided the plasmid and advice on performing this Barnase preparation scheme. This procedure uses a plasmid with a more tightly controlled promoter, in this case the $P_R$ phage promoter (10). Barstar is still present behind its natural promoter, and the plasmid confers ampicillin resistance to bacteria that have it.

This new procedure requires significantly less time to perform. To begin with it uses the commercially available terrific broth as media rather than the M9-minimal media. Cells were transformed in the same manner as described in the first procedure, an overnight 60 ml culture was grown up, and 6 L of terrific broth is inoculated as

described previously. The cells are grown for 16-20 hours in terrific broth at 37°C, no inducer is needed but ampicillin is added at the beginning and again at 8 hours. Twenty-five ml of glacial acetic acid is then added to each 2L flask, and they are allowed to shake for 10 minuets, before the cells are centrifuged as previously described. From this point the purification scheme is similar to the previous except that more SP-trisacryl M cation exchange resin must be used as there is much higher levels of expression and the volume must be doubled from 6 L to 12 L with distilled water due to higher salt concentration of the media. After being eluted from the matrix with high salt buffer the samples are lyophilized, and redisolved in water and urea and passed through a G-50 size exclusion column to remove salt and urea. The urea is needed to force all of the lyophilized Barnase back into solution. The final product is stored in solution at 4°C. Due to many problems with the expression of Barnase work has not progressed beyond this point, see results for details.

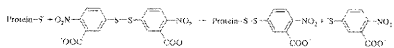
*Chemical Modification:* It is proposed to perform several modifications in an attempt to find one that was destabilizing enough to denature the protein under native conditions. Two modifications will be carried out to introduce an amide and carboxyl group into the core; it was also planed to introduce successively longer alky groups into the core as well as the small peptide glutathione. All chemical modification experiments need to be run in denaturing conditions to allow reactants access to the buried cysteine.

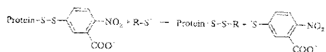
Iodoacetate and Iodoacetamide will be used to add the carboxyl and amide group respectively. The reactions will be carried out at pH 8.0, which is near the $pK_a$ of

cysteine, with 100 mM concentration reagents. The reaction is a standard double replacement $S_N2$ reaction with an iodide ion as the leaving group.

Two procedures were proposed for the addition of alkyl groups, both of which would take advantage of disulfide bond chemistry. The first and simplest scheme to modify Barnase A74C is to put it in a 2000 fold molar excess of R-S-S-R where R is the alkyl group that needs to be added. This huge excess is needed to make the reaction even approach completion. The reaction is R-S-S-R + Protein-$S^-$ $\leftrightarrows$ $^-$S-R + Protein-S-S-R. This reaction tends to take a long time to complete but can give yields as high as 99% (11). A further complication is introduced when the solubility of alky groups is considered. Large alkyl groups are not very water soluble, which means that this type of reaction would not be useful to add them.

The second procedure involves using activated mixed disulfides (AMD). Here the protein is first reacted with Ellman's reagent yielding the AMD of the protein. The driving force of the reaction is the release of the mesomeric thionitrobenzoate anion (TNB). The TNB has the nice property of absorbing light at 412 nm (yellow in the electromagnetic spectrum), thus allowing the reaction kinetics to be followed by spectrophotometery. The AMD of the protein consists of the protein disulfide bonded to another TNB molecule (see Fig 2). The TNB anions in solution are then removed and the alkyl thiol compound ($^-$S-R) is added in a 1-3 molar excess. They react with the AMD forming the R-S-S-Protein disulfide bond product. This reaction is again driven by the release of the TNB anion, which can be used to follow reaction progress. The lower reactant concentration makes this reaction useful for adding large fairly insoluble





alkyl groups to the cysteine, but this reaction has widely varying yields from about 30-90% (12).

Glutathione will be added in a manner similar to the first method prescribed for adding alkyl groups, as it is quite water-soluble and will form a disulfide bridge.

In any case Ellman's assay may be run after the reaction to determine the extent of modification, in the case of AMD method the TNB anions must be removed first. When Ellman's reagent is added, any free cysteines will react with Elman's reagent to release the TNB anion thus allowing for the quantification of the number of unlabeled proteins. After labeling it is necessary to remove reactants and urea from solution, so size exclusion chromatography would be employed.

*CD and Fluorescence Scans:* Then it is necessary to compare the labeled Barnase A74C with unlabeled Barnase. To do this CD and fluorescence spectrophotometery will be used. Wavelength scans will be made of unlabeled Barnase under both native and denaturing conditions. Scans will also be made of the labeled Barnase A74C under both conditions. It is hoped that the wavelength scan of one of the labeled mutants, under native conditions, will look similar to the unlabeled protein under denaturing conditions for both CD and fluorescence scans. Furthermore it would be hoped that all of the proteins appear similar under denaturing conditions.

*Size Exclusion Chromatography:* It is possible that no matching scan will be found, in which case new labeling experiments will need to be carried out, but if a match is found that modified Barnase A74C will be used to test the compressibility of the denatured state in aqueous solution. This will be done with a series of size exclusion chromatography (SEC) experiments. The column is filled with a resin that consists of

millions of microscopic beads with pores in them, smaller molecules move into the pores and must navigate these winding passages, whereas larger molecules bypass some pores and can go between the beads in a more direct route. In SEC the retention time of a protein is related to its size. More compact molecules are smaller and thus have higher retention times.

The SEC experiments will be carried out in increasing concentrations of trimethylamine N-oxide (TMAO), a good osmolyte. An osmolyte is a compound that does not solvate peptide bonds well and thus causes a contraction of proteins (13). As TMAO concentration increases so does the degree to which it contracts the proteins; if the unfolded form is very compact already, as native proteins are, there will be little difference in the SEC results as TMAO concentration increases. If the denatured state is extended as has been indicated in urea it should contract the protein a lot and increasing TMAO concentration will cause longer retention times. Furthermore standards will be run to determine the effective volume of the modified protein at each TMAO concentration. The standards will be used to get the retention time of proteins with know volume. This ends the description of the main experiments of the project (14).

There is one more occurrence that would be of great interest. The modified Barnase A74C may provide a means to isolate the burst phase intermediate and characterize it if it is stable. This could happen if the modification does nothing to hinder the burst phase step of folding, but does not allow folding to continue from there. This may allow for the direct observation of this intermediate ending the

controversy surrounding it. There is no way to make this occur it is simply and interesting possibility.

## CONCLUSION

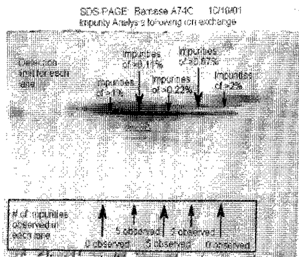
*Expression and Purification Scheme I Results:* Eight preps were done this way with some slight alterations to each in an attempt to improve yield. The largest yield was 12.5 mg and the smallest was 3.0 mg. It takes about 1 week to do a preparation, so at this rate it would take between 56 and 234 weeks to get the 700 mg of protein needed to start. Results are presented in table 1. There are several possible explanations for the low yields that were observed.

The fact that Barnase is a ribonuclease provides the most likley reason for the low yield. It does fold inside of the cell despite the presence of the phoA leader sequence, which confers it with activity. Barnase rapidly digests cellular mRNA shutting down metabolic functions. This leads to cell death. It is well established that the lac promoter is not very tightly controlled, thus there is some expression of Barnase even before induction with IPTG (10). It is possible that Barstar was not expressed at high enough quantities to counteract this Barnase, thus most of the cells would be dead by the time of induction. It is also possible that the Barstar gene was defective entirely.

It was believed at one time that Barnase yields were low because of a defective Barnase gene or promoter but gene sequencing determined that they were not the culprits.

Finally it cannot go unstated that minimal media has a detrimental effect on yield because; the bacteria must devote resources to making material that is supplied by

| Trial # | Yield (mg) | Notes |
|---|---|---|
| 1 | 3.3 | |
| 2 | 3.0 | |
| 3 | 4.5 | reduced preinduction temp. to 28°C |
| 4 | 6.7 | Induced at $OD_{600}$ of 1.9 |
| 5 | 3.1 | Induced at $OD_{600}$ of 2.4 |
| 6 | 8.6 | added case amino acids |
| 7 | 12.5 | only waited 36 hr after induction |
| 8 | 10.2 | only waited 36 hr after induction |


SDS-PAGE Barnase A74C 10/10/01
Impurity Analysis following ion exchange
Impurities of >0.11%
Impurities of >0.67%
Impurities of >1%
Impurities of >0.22%
Impurities of >2%
5 observed
2 observed
0 observed
5 observed
0 observed

richer medias. Adding case amino acids should help alleviate this burden, but this is not observed, so other culprits are responsible for the low yield.

Following purification each batch was assayed on an SDS-PAGE gel to determine the extent to which impurities were present as described in the methods section. Figure 3 shows 1 such gel, notice that no impurities are observed that are greater than 1%. It is desired that there be no impurities >2% thus the one step purification process is quite reliable.

*Expression and Purification Scheme II Results:* The first two attempts to use this new expression system met with frustration and no expression was observed. The third time this procedure was used expression was observed on a blue plate. The first 1/3 of this protein has been purified to the point of lyophilization. After elution from the column the absorbance at 280nm was determined and the amount of protein recovered was estimated to be 138 mg. It should be stressed that from only 2L of media already more protein has been obtained than in all of the previous preparations combined.

*Short-term goals:* The recovery of Barnase from the successful prep will continue, while site directed mutagenesis is performed to create the Barnase A74C mutant. This is necessary because the mutant has only been made to work under the conditions of the first procedure. The mutagenesis experiment will be carried out using the QuickChange Site Directed Mutagenesis Kit produced by Stratagene. Then it will be necessary to express and purify Barnase A74C before performing chemical modification. It is anticipated that the new preparation will work equally well for the mutant.

*Long-term goals:* The project will be completed. Chemical modification of Barnase will be carried out, and fluorescence and CD wavelength scans taken. If an appropriate candidate is found SEC will be performed as described in the methods section. The possibility of isolating the burst phase intermediate will be checked as well. Even more long term there is a possibility of replacing two of the three tryptophans with other amino acids and labeling the cysteine at position 74 with a fluorephore. This would allow fluorescence resonance energy transfer (FRET) experiments to be performed. This technique can be used to obtain the mean distance between the tryptophan and the fluorephore, even if the protein is denatured.

This work was greatly hampered by the pour results from the original expression system, but with this solved it is possible to continue with the experiments.

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I am currently a junior Biochemistry and Genetics Major. I take a lot of math classes in and may get a degree in mathematics as well. I have taught freshman chemistry lab classes and senior biochemistry lab classes here at Texas A&M University.

I have been working for Dr. Pace for nearly two years on various projects.

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