Inquiry: The University of Arkansas Undergraduate Research Journal

Volume 1 Article 9

Fall 2000

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Recommended Citation

Terry, A. (2000). Double Cysteine Mutations in Staphylococcal Nuclease: The effect of Artificially Introduced Disulfide Bonds on Protein Structure and Stability. *Inquiry: The University of Arkansas Undergraduate Research Journal, 1*(1). Retrieved from https://scholarworks.uark.edu/inquiry/vol1/iss1/9

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DOUBLE CYSTEINE MUTATIONS IN STAPHYLOCOCCAL NUCLEASE: THE EFFECTS OF ARTICICIALLY INTRODUCED DISULFIDE BONDS ON PROTEIN STRUCTURE AND STABILITY

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Abstract:

Since a protein's function depends on its structure, basic research in protein structure facilitates the solution of many practical problems, such as the synthesis of more effective medicines. With this larger goal in sight, the purpose of this research project is to understand better the chemical principles that underlie protein structure and stability. Disulfide bonds are a potentially stabilizing feature of many proteins. They may form between cysteine residues in close proximity to one another if the orientation is favorable. Often found in proteins produced by organisms that grow at high temperatures, disulfide bonds may anchor side chains together, making a protein resistant to thermal or chemical denaturation. In order to provide a better understanding of the stabilizing effects of disulfide bonds, disulfides are artificially introduced into the protein staphylococcal nuclease to create mutant versions of the protein. Wild-type S. nuclease has no cysteine residues, so disulfide bonds must be engineered by substituting cysteines for pairs of amino acid residues in the wild-type protein. To synthesize these double mutants, successive rounds of site-directed mutagenesis are performed on bacteriophage DNA using the Kunkel method. After transformation with the modified DNA, E. coli bacteria are used to synthesize the mutant proteins for analysis. Biophysical techniques such as solvent and thermal denaturation provide essential thermodynamic data for characterizing the stabilities of the mutants. On the basis of the data obtained from the S. nuclease mutants, generalized predictions about protein structure and stability can be established.

Anna Terry

Essay

The primary goal of this project is to understand better the chemical principles that underlie protein structure and stability. Since a protein's structure determines its specific function, knowledge of protein structure facilitates the solution of numerous practical problems. For example, the design and synthesis of new medicines, for example, depends in part on basic research into the mysteries of the protein folding process.

The 20 different amino acids are the basic components of proteins. A protein consists of a chain of amino acids in a unique sequence. Depending in part on this sequence, the chain folds into a unique functional form. Between different sections of the amino acid chain, widely separated in the linear sequence but close together in three-dimensional space, forces such as van der Waals interactions, hydrogen bonds, hydrophobic effects, and disulfide bonds help stabilize the three-dimensional structure of the protein.

Disulfide bonds, the primary focus of this research, are a potentially stabilizing feature of many proteins. They form between the sulfur atoms on two cysteine residues that are close to one another in three-dimensional space. Since it is an oxidation, the formation of a disulfide bond often takes place spontaneously in air if the orientation of the cysteines relative to one another is favorable. Disulfide bonds are relatively strong, and the crosslink they make in the chain creates a closed loop that cannot unfold. Thus they may stabilize the protein by anchoring side chains together, making it resistant to thermal or chemical denaturation. In terms of entropy, the presence of disulfide-bond forming cysteines lowers the number of possible final configurations into which a protein may fold during synthesis. Thus they greatly reduce the entropy of the protein in its denatured (unfolded) state. This causes the entropy change for the protein folding process to become more positive, which usually means that the folding reaction is favorable and that the functional (folded) state of the protein is more stable. Of course, this increased stability has an important biological purpose. Disulfide bonds are a common feature of proteins found in thermophilic organisms, such as the unique bacterial species that inhabit the superheated water near thermal vents in the ocean floor. To survive in hot conditions, these organisms need stable proteins resistant to thermal denaturation.

Previous attempts to introduce disulfide bonds into proteins have met with mixed success. It is not clear if the results are unsatisfactory because of poor site choice or because of stability factors. Because they induce permanent "kinks" in the protein chain, disulfide bonds may create an awkward functional form in the mutant protein. Thus they may actually disrupt the overall structure instead of stabilizing it as expected. The basic goal of this research is to determine whether disulfide bonds found at specific sites in proteins from thermophilic organisms can be transferred along with their stabilizing effect to a related protein that is less stable. If so, this would be encouraging information for those who wish to alter proteins to attain higher stabilities. If not, more detailed investigations will be needed to determine what other changes in nearby amino acids are required to reap the full benefit of the stabilizing effect of the disulfide bond.

The protein used for this project is staphylococcal nuclease, which occurs naturally in Staphylococcus aureus bacteria and has been cloned and expressed in Escherichia coli. S. nuclease is a good model protein for studies on protein stability for a number of reasons. It is smaller (149 amino acids long) and easier to purify than most other proteins. Its relatively simple structure allows the generalization of many experimental results to more complex proteins. It folds and unfolds reversibly, a property that is highly conducive to stability studies that utilize denaturation. The gene for S. nuclease is in an overexpressing system that makes it possible to produce mutant proteins in large yields. Finally, wild-type S. nuclease has no cysteine residues and thus no pre-existing disulfide bonds.

This project involves the mutation of the S. nuclease gene to produce proteins with two cysteine residues, which will be studied to provide a better understanding of how disulfides can be engineered into proteins to stabilize them. Disulfide bonds are artificially introduced into S. nuclease through the substitution of cysteines for two of the amino acid residues in the wild-type form. By introducing a disulfide bond at a specific site, the effect of the bond at that particular site on the protein's overall structure and stability can be estimated.

Possible mutants were chosen by comparing proteins whose genetic material is homologous to that of S. nuclease. Most of these proteins come from thermophilic organisms and have been sequenced as part of various genome projects. They are known to be particularly stable and to have pairs of cysteines, which means that they have the potential to form disulfide bonds, although it is not certain whether their structures actually include disulfide bonds. Since similar genetic sequences imply a similar structure, S. nuclease with cysteine mutations at the sites corresponding to those on the homologous proteins should also have this potential to form disulfide bonds, although this is not guaranteed. To ensure that the mutant pairs were favorably oriented for disulfide bond formation, the possible sites were also examined on a computer-generated model of S. nuclease.

Eventually, at least six double cysteine mutants will be made: A12C/L25C, L14C/V66C, V66C/V99C, A69C/A94C, I72C/V94C, and V74C/I92C. The letters to the left of the number in each abbreviation stand for the wild-type amino acid,1 and the numbers stand for the position in the amino acid sequence. More mutants will be added as information is obtained about each possible site of mutation. In order to make these double mutants, ten different single substitutions are needed at the following positions on the chain: 12, 14, 25, 66, 69, 72, 74, 92, 94, and 99. At least one mutant, I72C/V94C, will function as a control, since the mutated sites are too far apart in three-dimensional space for a disulfide bond to form.

In order to change the wild-type codon to the mutant codon, site-directed mutagenesis is performed on bacteriophage DNA using the Kunkel method.2 This method makes use of Kunkel DNA, which contains uracil instead of thymine. Modified oligonucleotides (relatively short chains of nucleotides) that contain the desired mutation of a specific site are enzymatically spliced into Kunkel DNA with ligase and polymerase. The resulting mutant DNA templates are transformed into a strain of E. coli known as DH5aF'. As the bacteria reproduce, the phage propagates itself by means of the bacterial genetic mechanism, and the desired mutant DNA is copied along with that of the phage. The reason for the presence of uracil, normally found only in RNA, is to ensure a more efficient phenotypic selection of the mutant. In other words, the genetic repair mechanism of the bacteria will not recognize the genetic material as its own DNA, so the mutations will be retained at a relatively high rate rather than reverting to the wild-type form.

After transformation, the mutant phage DNA is extracted, purified, and sequenced to determine whether it has the desired mutation. Preliminary results show that the Kunkel mutagenesis is working well for single mutants, with an efficiency rate of approximately 30% (the expected rate of mutation is between a quarter and a third of samples). DNA sequencing has also shown that the procedure yields samples that contain mixtures of mutant and wild-type DNA. Mutant DNA can be isolated from these mixtures by re-transforming and re-sequencing the phage DNA.

To make the double mutants, successive rounds of Kunkel mutagenesis and sequencing are performed until the desired mutant DNA is isolated. For double mutants, a more sophisticated technique is needed to retain the mutations during transformation. A different strain of E. coli called Cj236 is used, along with uridine and the antibiotic chloramphenicol to ensure a more efficient selection of cells that contain mutant DNA.

Finally, the entire S. nuclease gene is sequenced to confirm all the mutations and to make sure there are no unwanted abnormalities in the sequence. This is necessary because random mutations such as substitutions and deletions are quite common when the bacterial genetic repair mechanism is suppressed. After the correct sequence is determined, the mutant gene is incorporated into an E. coli plasmid, which is used to transform another strain of E. coli known as Ar120. The plasmid, along with the gene of interest, is copied with the bacterial DNA as the cells reproduce. At the same time, the genetic mechanism of the bacteria is induced to direct the synthesis of large amounts of mutant proteins for analysis.

After synthesis and purification of the mutant proteins, the effects of the mutations on stability and structure are characterized by various biophysical techniques. Ellman's assay is used to check for disulfide bond formation.3 Ellman's reagent, a spectroscopic indicator, will react only with free thiols (unbonded sulfurs). Since cysteine is the only amino acid that contains sulfur, and since there are only two cysteines in each mutant, the assay will give a negative result if disulfide bonds have already formed between the cysteines.

The stabilities of each of the mutants are characterized by solvent denaturation (with guanidine hydrochloride) and fluorescence-monitored thermal denaturation.4 The more stable the mutant protein, the higher the concentration of guanidine hydrochloride needed to disrupt its structure, and the more negative the free energy change for the denaturation will be. Both the oxidized form (the form containing the disulfide bond) and the reduced form (the form containing unbonded cysteines) will be analyzed using these procedures. As mentioned above, exposure to air should naturally produce the oxidized form. To prevent spontaneous oxidation during the analysis of the reduced form, reducing agents such as DTT (dithiothreotol) and TCEP (tricarboxy-ethylphosphine) are used. To provide further thermodynamic information about the mutant proteins, the solvent denaturation will also be carried out at varying temperatures.

If disulfide bonds fail to form in some mutants, it may be because the insertion of the bond at a specific site is too disruptive to the overall structure of the protein. Thus the single mutants that are required for the synthesis of doubles will also be analyzed as controls, in order to separate the effects of each single-site cysteine mutation from the effects of the disulfide bond formation. After statistical analysis of the free energy data, further predictions about protein structure will be generalized on the basis of the data obtained from the S. nuclease mutants. Eventually, the project may incorporate X-ray crystallography as a structural imaging tool.

Endnotes:

1 A = alanine, C = cysteine, I = isoleucine, L = leucine, V = valine

2 T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods in Enzymology*. 1987, 154, 367.

3 P. W. Riddles, R. L. Blakely, B. Zerner, *Methods in Enzymology*. 1983, 91, 49.

4 Fluoroscopy is possible because S. nuclease has a single tryptophan residue, which responds to fluorescence. The method is taken from M. P. Byrne, R. L. Manuel, L. G. Lowe, W. E. Stites, *Biochemistry* 1995, 34, 13949.

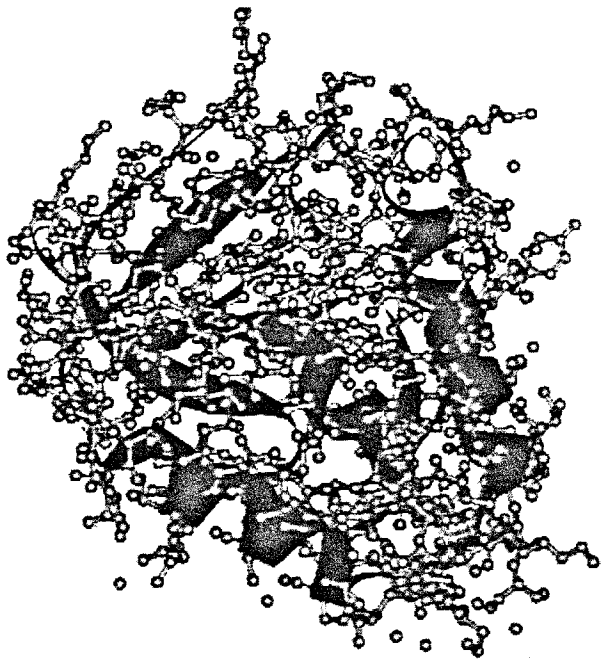

Figure 1. Wild-type staphyloccal nuclease

Faculty Comments:

Wesley E. Stites, Associate Professor of Chemistry and Ms. Terry's mentor, writes:

> Anna has been working in my lab since 1997 and I have had an excellent opportunity to evaluate her in that time. Anna is double majoring in two unrelated fields, Chemistry and German, and is doing superbly in her courses. She is also a serious student of music and considered for a while majoring in that area. She has taken an impressively heavy course load during her time here and maintained a 4.00 GPA. Simply put she is an exceptionally bright and multi-talented individual who will succeed in whatever career path she follows.
>
> In her work in my lab Anna is exploring the role of disulfide bonds in stabilizing protein structures. This project is not only intriguing intellectually, but is also deals with a question of considerable practical importance as methods to improve protein stability are of great interest to industry. Anna has made a series of disulfides through site directed mutagenesis and characterized their effects on the stability of our model protein system. This will be of great utility in future efforts to develop models of disulfide effects.

Collis R. Geren, Dean of the Graduate School and Associate Vice Chancellor for Research, has this to say about Ms. Terry:

> What should I say about a student who has two perfect scores on the SAT and a perfect score on the ACT, who has maintained a flawless record in her four years at the University of Arkansas majoring in chemistry, who is an impressive concert violinist, who speaks German fluently, and who reads Melville aloud with her friends. To say that Anna Terry is an academic jewel is an understatement.
>
> Ms. Terry combines intelligence with curiosity and industry, a combination that is extremely appealing. She is a Sturgis Fellow, and a good choice it was. Anna Terry excels at everything she does. This sounds like a cliche, but it fits her perfectly. Her reputation as a violinist is such that our chancellor went to a concert of hers during his first year on our campus and was amazed by the quality of the performance. What is more amazing is that Ms. Terry is not pursuing music as vocation. It is simply one of the many things she loves to do and does extremely well.
>
> Faculty members look forward to working with Ms. Terry. John Hehr, Associate Dean of the Fulbright College and Professor of Geosciences, considers himself her mentor, a position he requested, and he is just one of many. She is also very accomplished in her field of chemistry. She has received every campus award we have to offer. She was also recently awarded a Science Information Liaison Office Undergraduate Research Fellowship for her proposed project on double cysteine mutation in staphylococcal nuclease. As a sophomore she received an honorable mention from USA Today. She has just learned that she has also been selected as a Barry Goldwater Scholar. This is the premier undergraduate award for students in mathematics and science and is given specifically on the basis of the student's research record. Ms. Terry embodies everything such honors stand for: intelligence, integrity, wit, creativity, determination, and industry.
>
> I have known Anna Terry for four years, and I have come to admire her very much. She is meticulous in her preparation. She is celebrated by Professors Sakan and Stites for the work she has done in their labs because she does exactly what she promises to do. She is ambitious in her work and more, and she will persevere until she has successfully and intelligently completed short-term projects or long-term goals. In short, she is terrific.

Donald R. Bobbitt, Professor and Chair of the Department of Chemistry and Biochemistry comments:

> I am writing to offer my highest recommendation for Ms. Anna Terry. I have known Ms. Terry for approximately four years, having served as her mentor and advisor. I first met Ms. Terry while serving as a judge at a regional High School Science Fair. It was clear to me then, as it is now, that Ms. Terry stands out in a crowd, even when the cohort is a group of gifted and talented individuals. From my observations of her at the University, it is evident that my initial expectations for her were correct
>
> Ms. Terry is one of the most intellectually gifted students I have met in over fifteen years at the University of Arkansas. The list I am comparing her to includes former scholarship recipients. In addition to her innate ability, she also has an unusual degree of persistence and competitiveness. She simply will not leave a problem without mastering it. It is obvious from her vita that her abilities are not one-dimensional and limited only to course work; rather, she performs at an exceptional level in a number of different fields including music.
>
> Although I do not have direct knowledge of Ms. Terry's ability in the research laboratory, I am confident she has the tools to be extremely successful. Ms. Terry has the intellectual resources and confidence to attack difficult and involved problems, and the perseverance to carry through the problem to completion. In summary, Ms. Anna Terry is a student of immense promise and ability.