Crystal Structure of the ATPase domain of the molecular chaperone Hsc66

Abstract

The molecular chaperone Hsc66, consisting of a substrate binding domain (SBD) and an ATPase domain, is a member of the heat shock protein 70 (hsp70) family. Hsc66 is important in iron-sulfur-cluster biosynthesis. These clusters are important in many functions vital to all living organisms and the mechanism for its synthesis is unknown and presumably universally conserved. Thus, the study of Hsc66 will contribute to the understanding of this important biosynthesis. In the Vickery Lab, the SBD domain has been recently crystallized; but the ATPase domain has not, though it has been attempted. It would be of interest to this lab as well as to the overall study of iron-sulfur biosynthesis to elucidate how these two domains come together. A crystal structure of the ATPase domain attached to the SBD would lead to an understanding of the observed properties of Hsc66 (2,3,4,5,6). A truncated form of Hsc66 has been designed specifically for this end, attempting to keep possible conformational variations to a minimum. This project involves protein engineering, expression, and purification; biochemical assays to determine the importance of the parts of the protein cleaved off; and finally structure determination.

Introduction

Purpose- The structure/function of Hsc66 will be investigated in this project. In the end, ideally, the relationship between an ATPase domain, at the N-terminus, and a SBD of Hsc66 will be elucidated by the determination of the structure of an N-terminal cleaved form of Hsc66 (fig 1) through crystallography and possibly NMR. If this fails, an attempt will be made to determine the structure of just the ATPase domain of this N-terminal cleaved Hsc66 through crystallography (fig 1). As this requires constructing a truncated form of Hsc66 this project will have 3 parts: Part I will involve engineering, expressing, and purifying the truncated version of Hsc66; Part II will involve performing assays to determine the function and importance in activity of the cleaved amino acids; Part III will involve structure determination.

Background and Rationale- The 66-kDa Hsc66 is a member of the hsp70 family of ATP dependent molecular chaperones. The hsp70 family is a large group of relatively conserved proteins expressed in organisms from prokaryotes to eukaryotes. Although called heat shock proteins many members of hsp70 are not induced by heat shock but are important in normal cell function; as is the case with the constitutively expressed Hsc66 (6). The gene encoding Hsc66, *hscA*, is within the gene cluster, *iscSUA-hscBA-fdx*, which encodes for related molecular chaperone proteins important in iron-sulfur cluster assembly and regulation.

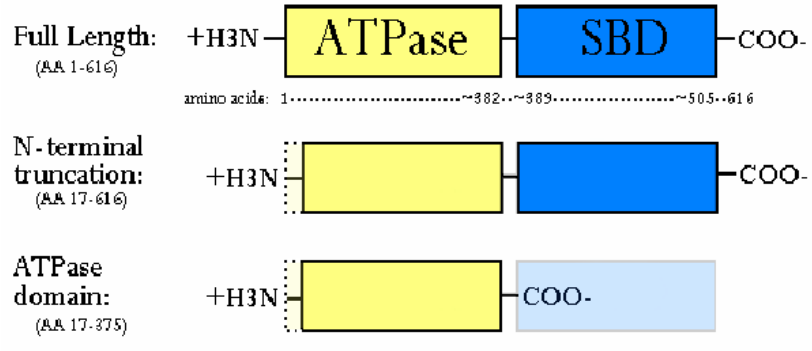

Figure 1. Truncations of Hsc66 ATPase and SBD domains. *Initial studies and crystallization will be performed on the N-terminal truncated version (at amino acid 17) with the SBD. If the N-terminus truncation is found to cause minimal loss of function and crystallization proves difficult, the truncated ATPase domain alone will be expressed for crystallization.*

The precise role that Hsc66 plays in iron-sulfur assembly is unknown; however it is known that it plays a role and that it functions with other proteins encoded by its gene cluster such as IscU and Hsc20 (2). It is likely that it regulates the iron-sulfur cluster formation on IscU, which is proposed to act as an iron-sulfur scaffold (2).

The study of the biosynthesis and regulation of iron-sulfur clusters is of great interest as these clusters are found in all living organisms and are involved in vital functions such as electron-transfer, catalysis, and gene-

regulation; and, importantly, its mechanism of biosynthesis is highly conserved and remains unknown. Consequentially, the study and understanding of these *E. coli* iron-sulfur proteins will shed light on all other iron-sulfur cluster assemblies in *E. coli and* other prokaryotes as well as those in the eukaryotic mitochondria. A homologue of Hsc66, DnaK, not involved in iron-sulfur biogenesis but well studied (1,6) can shed light on Hsc66 and can be used for comparison in determining a logical construct of Hsc66 for crystallization.

DnaK, like Hsc66, consists of an SBD and an ATPase domain. DnaK and Hsc66 have similar, 45% (6), conserved amino acid sequences and consequentially similar allosteric behavior: ATPase activity in the nucleotide binding domain causes a relaxed state in which the SBD has an increase in affinity for bound peptide; whereas when ATP is bound a tense state exists in which the SBD has a low affinity for peptide (5). This allosteric relationship is complex; for example it has been shown that in the presence of IscU the ATPase function of Hsc66 is induced, increasing binding affinity for IscU at the SBD (2). Determining Hsc66's structure may elucidate exactly how this relationship is transmitted between domains.

The structure of the SBD and ATPase domain (bound with GrpE) of DnaK have been determined through crystallography, but separately (1, 7). So the structure of both together still needs to be found for better insight into these homologous proteins' observed characteristics. Thus it would be ideal to determine the structure of the ATPase domain attached to the SBD. Recently the crystal structure of a truncated version of the Hsc66 SBD with a short peptide bound was determined in the Vickery Lab. However, the full size ATPase domain has been difficult to crystallize with any success. This could be due to certain parts of the protein being too motile, especially the long N-terminus described below. So it may be advantageous to design a construct that truncates unnecessary portions of the ATPase domain and to bind it with ADP to keep it in as few conformations as possible. Then there would be a possibility that both domains could be crystallized together.

As expected, Hsc66 and DnaK also have their differences, specifically, in the N-terminal amino acid sequence; in their interaction with other proteins; and in their ADP dissociation rates (2, 5). The N-terminus (ATPase domain) of Hsc66's amino acid sequence is longer by about 16 non-conserved amino acids than DnaK's (fig 2a). In addition, in kinetic studies Hsc66 released ADP much faster than did DnaK (5). Also, DnaK interacts with GrpE (a nucleotide exchange factor), which has no homologue in the Hsc66 system; however with GrpE bound DnaK's ADP dissociation becomes similar to that of Hsc66. Thus in view of these differences, it is possible that the extra 16 amino acids of Hsc66 at the N-terminus of the ATPase domain may assume a function similar to the separate protein GrpE in DnaK. It is also just as likely it has other functions. It is very unlikely this N-terminal segment is of no consequence to Hsc66, and so steps need to be taken to determine, before the lengthy process of structure determination begins, whether the truncated protein is at all similar to wild type Hsc66 and thus whether a structure of this truncated protein is even worth while pursuing.

The sequence of the extra length of the N-terminus is not conserved, upon a precursory check, between the few homologous hsp70s that do have longer ones. In addition there are other large segments of the amino acid sequence that differ between the homologous proteins, not just at the N-terminus, and it is conceivable any of these other segments could be responsible for the observed differences in behavior. It would be safe to assume that this proposed truncation of the first 16 amino acids would not have much of an effect on Hsc66.

Thesis- The first 16 amino acids of Hsc66 most likely do not have function and can be safely cleaved with no effect. However, if they do have a function this cleavage along with proper biochemical assays will help determine it. This cleavage as well as binding a short peptide and ADP will increase the probability of obtaining a crystal useable in crystallography studies for the ATPase and SBD domains of Hsc66 together. This structure should be valuable in understanding structure/function relationships in Hsc66 as a whole.

What I Have Done/Concerns- I have designed the construct and have amplified the N-terminal cleaved DNA which is now ready to be expressed. Wild type Hsc66, the full-length ATPase domain, and separate SBD have expressed in large amounts in the lab before and its protocol is well known; expressing a truncation of about 16 AA should not be too much more complicated. Assays for Hsc66 activity are currently being performed in the lab, and I can learn these easily. During the summer I performed crystallization on another protein of the same gene complex: IscS+IscU, so I am familiar with crystallization procedures.

It is possible the protein may not express properly, in which case I would have to reconstruct the truncation until I run out of options at which point I would drop the project altogether. The loss of function with the truncation would hinder my progress towards structure determination, however it would lead to an understanding of the importance of the first 16 amino acids of Hsc66. This understanding would be significant in its own right. Nothing is guaranteed in crystallization; however, the possibility of NMR studies makes successful crystallization not completely necessary for my final goal of structure determination.

Materials and Methods

PART I

Construct- hscA is available in a plasmid (ptrc99a) designated ptrc66 from past cloning. The 16 AA from the N terminal of Hsc66 will be cleaved via PCR cloning. 5' primers with the mutations necessary for the truncation were designed (fig 2a). Q17 was converted into a methionine and R18 converted into an alanine. This provided an NcoI site, which can be inserted into ptrc99a for proper expression. This design also gives an amino acid with a small side chain, alanine, adjacent to the methionine, as is in the wildtype (see fig 2a), and thus the methionine should be cleaved post-translationally, as it is naturally. This location was chosen as it preserves the heavily conserved region starting at L20; but at the same time leaving a positively charged side chain in R19. This positive group appears upon a precursory evaluation of the DnaK ATPase domain crystal structure (the corresponding amino acid would be K3 in DnaK) to have possible interactions with negatively charged Q137 and Q150. Hsc66 also has negatively charged amino acids in corresponding proximity, in Q148 and D150. R19 of Hsc66 was kept for these possible interactions; and even if there is no interactions (as this is all speculative), it does not provide a large tail for wagging (1 AA from conserved AA L20) that could disrupt proper crystal formation. The 3' primers for the inclusion of the SBD domain have already been designed from past cloning of Hsc66. After PCR cloning this fragment was reinserted into ptrc99a (at NcoI and XbaI); hereon called ptrc66 @17. In addition the 3' primers, to be used with the above truncation to produce just the ATPase domain by itself, have been designed (fig. 2b). For this, D375 was converted into a stop codon. This amino acid was chosen because its negatively charged R group will be mimicked by the newly created negatively charged free carboxyl terminus.

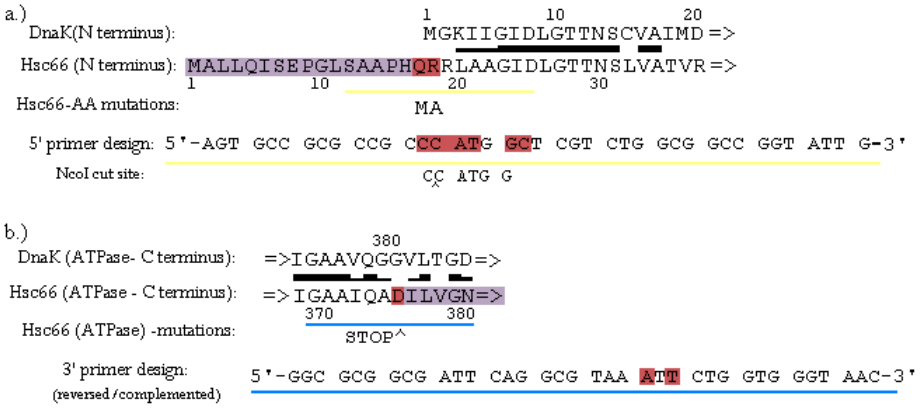

Figure 2. *Amino acid sequences of Hsc66 vs DnaK / primer sequences.* Black bars indicate conservation of AA (full bar = complete, half bar = similar side chain). Red indicates alterations made for truncation. Purple indicates absence from the designed protein. a.) N terminus and 5' primer. b.) ATPase – C terminus and 3' primer

DNA amplification and Protein Expression and Purification – The DNA for the N-terminal cleaved Hsc66 will be cloned using PCR cloning followed by ligation into ptrc99a. Z-competent ptrc66 @17 transformed K12 strain DH5α *E. coli* will be used to amplify the DNA and to determine if protein expression is possible. Protein expression will be performed using a mutant *E. coli* lacking the wild-type *hscA*; by retransformation

with the ptrc66 @17 from the previous step. A purification protocol is already in place for wild-type Hsc66 (6), involving a series of chromatographic columns: hydrophobic chromatography and anion-exchange chromatography. The cleaved Hsc66 (16 AA shorter) should be able to be identified by comparison to wildtype Hsc66 on SDS-PAGE. Before a large-scale prep is performed the sequence of the DNA in the ptrc66 @17 will be sequenced by sending some of the amplified DNA to Laragen in order to confirm it is the truncation I want.

PART II

Assays – An ATPase assay can be performed to determine steady-state ATPase rates and compared to wildtype ATPase activity as described (6). The rates of ATP association and ADP dissociation can be determined as described in (5) to see if the 16 amino acid N-terminus has an effect like GrpE on DnaK. If required an SPR analysis can be performed to determine if Hsc20 and IscU binding affinity to Hsc66 is retained as described in (2). In addition isothermal titration calorimetry can be used to find the Hsc66 equilibrium binding constants for ADP as described in (5).

If through the assays it is shown that the N-terminal 16 amino acids are unimportant, I can proceed to attempt to crystallize the truncated-ATPase+SBD Hsc66. At the same time I may be able to proceed with NMR studies. If crystallization of the ATPase+SBD Hsc66 appears unsuccessful I can attempt to express and purify just the N-terminal cleaved ATPase domain. Most likely this step will be performed as crystallization trials for the ATPase+SBD is proceeding, once it has been determined that the N-terminal truncation has no effect on function.

If it is shown that the N-terminal 16 amino acids are important in Hsc66 function, I can redesign the construct with a longer N-terminus to determine exactly what portion is important. Once the truncation behaves similar to wild type I can proceed with crystallization.

PART III

Crystallization – Crystallization will be attempted using Hampton Screening Kits. I will attempt crystallization of the ATPase + SBD Hsc66 with bound Mg^{++} + ADP + Pi and bound peptide –(LPPVK Ref 3). If just the ATPase domain is attempted to be crystallized, I will use Mg^{++} + ADP + Pi.

X-ray Crystallography / NMR Data Analysis – X-ray crystallography can be performed on campus or off campus for higher resolution. NMR studies will be performed on campus. 15N-, 13C- and D2O-labeled HscA, under the same construct, will need to be expressed. Since the structure for the SBD of Hsc66 has been determined and the ATPase domain of Hsc66's homologue DnaK has been determined, homology modeling can be used and I have been assured that x-ray data analysis should not be too complicated and should be straightforward to learn. In addition, if NMR studies are performed, the backbone structure of the protein can be easily taught how to be determined from the data.

Responsibilities

I will be doing nearly all of the work described above autonomously. The different protocols for Part I will be supervised by Dennis Ta, in the Vickery Lab. I will learn the assays for Part II from Tim Tapley, a grad student in the Vickery Lab. For Part III, if the NMR studies are performed, the data collection will be done by Dr. Melanie Cocco and I will be taught by her how to analyze this data. If crystals are obtained, Dr. Jill Cupp-Vickery will perform the data collection and will teach me how to analyze the data. The concept of this experiment came from Dr. Larry Vickery; and future guidance on this project will be provided by Dr. Larry Vickery.

Timeline

Fall

October - Construct truncation of Hsc66.

November - Express and Purify the N-terminal truncated Hsc66.
December - Perform assays on Hsc66
Begin crystal screening.

Winter

January - Continue crystal screening
Express and Purify the N-terminal truncated ATPase domain of Hsc66.
Try NMR studies?

Spring

March - Data analysis (x-ray or NMR)
Continue crystallization attempts with both versions of truncated Hsc66.

Budget**

ITEM	Cost $
DNA (oligo primers, ptrc99a…)	100
DNA sequencing (~1973 bp) (2x)	40
Molecular Biology Reagents (XbaI, NcoI, buffers, PCR reagents, ligase)	100
Bacterial Growth media (TB + ampicillin / plates)	100
Chromatography materials cc(buffers, salts)	100
Crystallography materials (salts, buffers, precipitants, screening kits, crystal boxes, tools)	200
Assay materials	100
Travel expenditures (trip to Stanford synchrotron for high res. xtal data)	300
TOTAL:	**1040**

**(As this project is within the goals for which the Vickery Lab receives NIH funds, some of the costs may be defrayed by the lab's grant.)

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