The Consensus Ankyrin Domain as a scaffold for protein engineering: Implications in cellulose to ethanol conversion

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Introduction

Fossil fuel reserves (the most common energy source used) are being depleted and their depletion has negative environmental, political and economic consequences. Production of ethanol from cellulose (a polymer composed of β-D-glucose - the main structural component of plants) helps in solving these problems, since biomass constitutes one of the most available energy source, and since the CO2 produced by ethanol processing and combustion is captured from atmospheric CO2 by photosynthesis. However, there are major barriers to the cost-effective production of biofuel that have to be overcome. Current problems include low activity of the celullase enzymes used in this process, and its high production costs.

The cellulosome is an extracellular complex that degrades cellulose and other polysaccharides present in plant's cell wall. The cellulosome is active in the absence of the cell and functions in both aerobic and anaerobic conditions. A scaffolding protein holds together the various enzymes, through cohesin-dockerin interactions, and as a result of this proximity the product of one enzyme can readily be used as the substrate of the next enzyme, promoting a greater efficiency in cellulose breakdown. Each enzyme that constitutes the cellulosome is composed of a catalytic and a docking unit that allows it to interact with the scaffolding protein. However, this mode of polyvalence lacks specificity on the spatial arrangement of the several cellulases. Our goal is to use the consensus ankyrin repeats as a scaffold which will allow us to increase or decrease the spacing between cellulases as necessary, and to have distinct stereochemical orientations.

Results

The consensus ankyrin is extremely stable. To graft cellulose degrading enzymes into this template, we have designed a new repeat that has substitutions on position 13 (R13). We have previously shown this position in the Ankyrin repeats to be tolerant to loop and domain insertions. For a construct with five repeats with R13 positioned in the middle, the free energy is of 14.3 kcal/mol as shown by guanidine denaturation experiments. This represents a loss of ~ 6kcal/mol relative to wild type repeat protein.

We have used the consensus domain as a scaffold to which we can attach a variety of different cellulases from extremophile organisms, such as *Clostridium thermocellum* and *Thermotoga maritima*. One determinant factor in choosing these enzymes was for them to have their termini in close proximity (between 10-20Å apart) so that the ankyrin repeats would remain as a single unit. We have successfully purified 3 cellulases inserted into the 3rd repeat in a 5-repeat array which have also been purified as a single domain in the lab. All the constructs are folded and the temperature melts transitions are sharp with the midpoints spanning from 60 to 75^{o}C, showing that the protein is unfolding in a concerted mechanism. These temperature melts followed by circular dichroism of the Ankyrin-Cellulase constructs compared with their single counterparts showed that the insertion into the ankyrin is not destabilizing. The cellulolytic activity was measured using the DiNitroSalicylic acid method (DNS). We have adapted the DNS assay to a high-throughput format in our lab. All the constructs are able to degrade

both soluble and insoluble cellulose (CMC, Avicel, PASC and Filter paper were tested). In all the cases the insertion into the consensus ankyrin was either neutral or improved the activity at the conditions tested. For the single construct with highest activity we have increased its thermal stability and seen a 2-fold increase in cellulolytic activity at high temperatures. A cellulose binding module has also been purified as a single domain and attached to the N-terminus of consensus ankyrin. Both the CBM and CBM-Ank showed the ability to bind microcrystalline cellulose in the lab. Surprisingly, attaching a cellulose binding module at the N-terminus of the cellulase-Ankyrin constructs seems to inhibit the cellulolytic activity. We have also been able to purify 5 constructs with two cellulases inserted into a 7 repeat consensus ankyrin with 3 repeat spacing in between. Two of these double constructs have the same endocellulases but with the domains order swapped in the anchoring protein. The order of the domains plays an important role in the thermal stability however the cellulolytic activity profile remains about the same. This is indicative of having both cellulase domains folded but having the ankyrin repeats partially folded. One of these double endocellulase constructs shows synergism. These two ankyrin-cellulase showed synergism in trans, however the synergism in cis is even higher confirming that the proximity does play a role in this case. We have been determining the best pH and temperature for which these proteins are more active. The best pH in all instances is of about 5 which is in accordance with the underlying cleavage mechanism. The best temperature varies according to the construct. In all instances the highest activity was seen above 50^{o}C. In some cases these enzymes were still active at 95^{o}C. We are currently cloning exocellulases into the consensus that we have shown to be active as a single domain. The ultimate goal is to achieve increased synergism between two and more cellulases embedded in the consensus ankyrin domain.

Conclusion

Our results so far indicate that consensus ankyrin repeats are a promising target for cellulase engineering. These repeats are stable and have a regular spacing that can be altered by adding or removing repeats without significantly destabilizing the protein. This is ideal for designing a linear scaffold on which to graft the enzymes required for a biochemical pathway such as the conversion of cellulose to ethanol, in a precise spatial arrangement. This new system can improve the yield of the cellulose to ethanol reaction improving its economical viability. Finally we plan on evaluating the system using other type of non-ideal substrates and determine its advantages and disadvantages when compared to existing architectures.