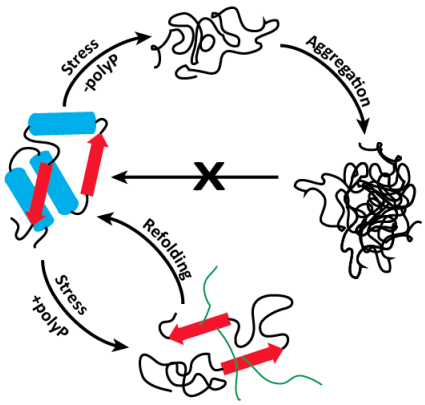
Polyphosphate: Elucidating the Protective Mechanism of an Ancient Chaperone

Overview: I will study how inorganic polyphosphate (polyP), a chain of up to 1000 orthophosphate monomers, protects proteins against aggregation. Although we have shown that polyP acts as a chaperone to prevent protein aggregation[1], the exact mechanism of protection remains unknown. Initial results indicate that polyP may interact with β-sheets and keeps proteins in a refoldable conformation[1,2]. Therefore, I hypothesize that polyP stabilizes proteins by selectively binding β-sheets. This prevents aggregation and maintains proteins in a refoldable conformation. To test this model (see below), I propose the following aims:

1. To test whether polyP directly binds proteins *in vitro*, I will use fluorescence anisotropy, dynamic light scattering (DLS), and a malachite green assay.
2. To test whether polyP selectively stabilizes β-sheets *in vitro*, I will use circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy.
3. To test whether proteins protected by polyP can be refolded, I will use CD, NMR, and activity assays.

Since polyP is predicted to have been present on Earth since pre-biotic times[3], it is thought to have acted as one of the first chaperones in the origin of life. Furthermore, potential industrial applications of polyP include drug development and protein purification.

Background: To execute their intended cellular functions, proteins must be properly folded. To ensure proper folding, cells maintain stable internal conditions. When cells deviate from these stable conditions, as caused by heat or oxidative stress, for example, proteins begin to unfold. These unfolded proteins then begin to accumulate, losing their function and forming large aggregates. This irreversible process of aggregation places a heavy tax on cells and can even lead to cell death[4] (see model).



Model

To combat aggregation, cells have developed preventive mechanisms. Molecular chaperones, such as GroEL and GroES, protect cells by segregating and binding unfolded proteins, thereby preventing aggregation. Furthermore, molecular chaperones keep proteins in a refoldable conformation. Once stress conditions subside, refoldases, such as the DnaK/DnaJ/GrpE system, can refold proteins to restore native function[5]. Recent work in the lab has identified polyP as a novel and highly effective chaperone, preventing aggregation under both heat and redox stress conditions[1]. Initial work with luciferase has shown that upon heat stress in the presence of polyP, luciferase assumes a β-sheet conformation and can be refolded to regain some activity[1].

This knowledge has led to the three claims of my hypothesis. Since molecular chaperones generally act through direct interactions, I propose polyP will also act through direct interactions. Additionally, our initial findings lead me to believe that polyP selectively stabilizes β-sheets and keeps proteins in a refoldable conformation. To test these claims more rigorously, I propose three separate aims:

Aim 1: Confirm that polyP directly binds proteins

I will use three separate standard techniques to test this hypothesis: fluorescence anisotropy, DLS, and a malachite green assay. For these assays, luciferase will be the binding partner of

polyP due to its previously described high degree of protection[1]. For fluorescence anisotropy, I will measure the anisotropy of fluorescently-tagged (e.g. rhodamine) polyP of various defined lengths[6] in the presence or absence of luciferase. Due to an increase in hydrodynamic radius (R_H), the protein-bound polyP spins more slowly than free polyP. For DLS, I will measure the light scattering of free protein and polyP-bound protein. This time, an increase in R_H due to polyP should lead to a broadening of light scattering intensity. Finally, I will use a malachite green assay to quantify the amount of polyP bound to luciferase. In the presence of free phosphate, malachite green has an absorbance peak around 630 nm. Free phosphate can be created and quantified upon the addition of exopolyphosphatase (PPX) to polyP[1,2]. PPX added to free polyP should return a malachite green signal that is stronger than PPX added to a polyP-protein complex, as some phosphate in the latter is secluded from PPX. While this assay will not return an exact number of phosphate monomers bound to luciferase, it will show whether small or large portions of polyP bind luciferase.

Aim 2: Test whether polyP selectively stabilizes β-sheets

I will test this aim by using standard CD and NMR spectroscopy to record 2° structure[1,2]. As experimental proteins I will use arc (predominantly α-helices), Human cystatin C (HCC; predominantly β-sheets), and citrate synthase (CS; mixed 2° structure elements). I expect to see retention of 2° structure in CS and HCC, but not in arc upon the addition of polyP. As a negative control, I will use the 46 C-terminal residues of α-synuclein that are intrinsically disordered with no structure to show that polyP does not induce β-sheet formation. For a more structural view, I will employ 2D NMR. By looking at the retention of cross peaks, this technique will allow me to see precisely which parts of the proteins are protected by polyP.

Aim 3: Validate that proteins protected by polyP can be refolded

To test my third aim, I will use CD, NMR, and activity assays. CS and luciferase with His6-tags will be exposed to heat and oxidative stress (e.g. bleach and H_2O_2) in the presence and absence of polyP. After being stressed, the proteins will be returned to favorable conditions in the presence of the refoldase system DnaK/DnaJ/GrpE. In the CD experiments, I expect a return to native structure of CS and luciferase. Since the refoldases would also contribute to the CD signal, CS and luciferase will be re-purified using the attached His-tags prior to spectroscopy. Similarly, NMR will be used to ensure proper refolding has occurred. As a folded protein itself does not necessarily confer function, I will also run activity assays to show that function has been regained. For CS, citrate can be converted into oxaloacetate and acetyl-CoA, the latter of which can be fluorescently detected. For luciferase, the addition of luciferin and ATP will lead to fluorescence. By showing that CS and luciferase refold, I am simultaneously showing that they do not irreversibly aggregate in the presence of polyP.

Significance: One of the classic chicken-or-egg problems in biology is whether enzymatic proteins or chaperones came first. Since polyP is predicted to have been around since pre-biotic times, it could have played a significant role in biogenesis. A clearer understanding of how polyP works will thus help us better understand how life originated. Further, polyP is of industrial interest. Inhibiting polyP activity in bacteria could make them more vulnerable to drug treatment. Lastly, overexpression and purification yields of unstable proteins can be increased by polyP's stabilization effects.

References: [1] Gray *et al.* (2014) *Mol. Cell* 53:689. [2] Wagner *et al.* (2014) *in prep.* [3] Kornberg *et al.* (1999) *Annu. Rev. Biochem.* 68:89. [4] Winter *et al.* (2008) *Cell* 135:691. [5] Bardwell & Jakob (2012) *Trends Biochem. Sci.* 37:517. [6] Choi *et al.* (2010) *Biochem.* 49:9935.