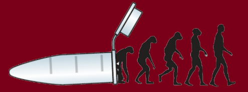
Did Early Functional Proteins Use Metal Ions to Bind ATP?



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Abstract

In early life forms, the first proteins were likely composed of a limited set of amino acid building blocks. Previous work in the Seelig lab has produced random libraries of 85 amino acid-long proteins with ATP binding affinity, made from varying subsets of the 20 modern amino acids. By characterizing these proteins' binding mechanisms and properties, we hope to gain insight into the nature of early functional proteins. Here, we took initial steps to analyze the metal ion affinity of one such protein composed of five types of amino acids. Further analysis will confirm the dependence of ATP binding on metal ions and extend these investigations to other selected protein variants.

Introduction

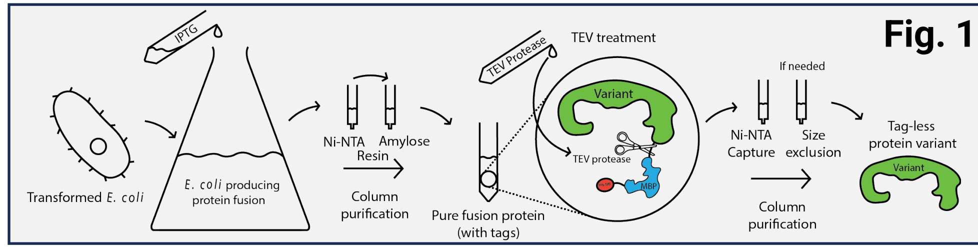
Proteins are one of the four main macromolecules in all cells and serve as the major functional players in nearly every aspect of life. However, proteins likely arose with a limited subset of the modern 20 amino acids. Trifonov et al.[1] analyzed more than 60 relevant criteria to identify a likely consensus order in which amino acids were added to the genetic code. Using this consensus order, Newton et al.[2] created four libraries of random proteins composed of only 5, 9, 16, or all 20 predicted earliest amino acids. Using mRNA display, these libraries were selected for proteins that bind to ATP—the energy currency of life—to yield four groups of functional proteins.

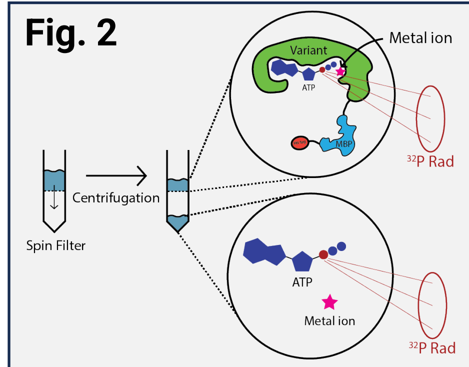
By comparing the mechanisms of ATP binding between these groups, we hope to better understand how the earliest functional proteins may have functioned and evolved. Here, we have attempted to characterize the effect of divalent metal ions on the ATP-binding affinity of our model proteins. Since the five amino acid library lacks both aromatic and positively charged amino acids, we expect its ATP binding mechanism to significantly differ from that of modern proteins, which utilize both for π-π and cation–π interactions with ATP's adenine base. **We hypothesize that magnesium ions, commonly used in nature to coordinate the phosphate groups of ATP, may also be utilized by our model proteins.**

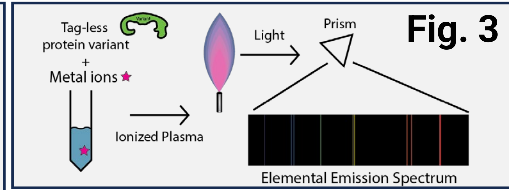
Methods

To produce protein for analysis, protein was expressed in RosettaTM(DE3)pLysS *E. coli* using a pET expression vector with IPTG induction of the lac operon. Each protein was expressed as a fusion with maltose binding protein and 6xHis purification tags connected by a cleavable TEV protease recognition site (see Fig. 1). Cells were lysed by sonication and centrifuged at 30k x g. Protein fusions were purified by both Ni-NTA and Amylose resin columns. Purity and yield were assessed with A280 UV absorbance and LDS-polyacrylamide gel electrophoresis (PAGE). Purification tags were cleaved away with TEV protease and captured with Ni-NTA resin (Fig. 1). Free protein was quantified via Bicinchoninic Acid (BCA) assay. For characterization of proteins, a ^{32}P radioactive ATP binding assay was used to test the binding of protein variants to radioactively labelled ATP. Samples were incubated with a mix of 7 divalent metal ions (Ni^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Mg^{2+}) for one hour. Samples were then fractionated using ultrafiltration spin filters at 16873 rcf for one minute.

Methods cont.







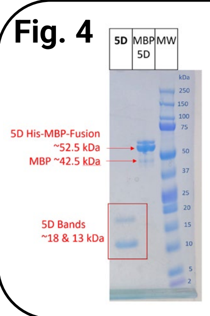
Methods cont.

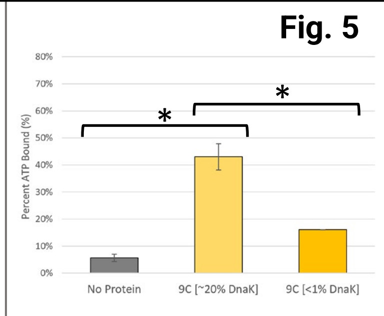
After centrifugation, the relative proportion of ATP in the retentate and filtrate was used to determine ATP binding affinity (Fig 2). After metal treatment, samples were dialyzed against buffer containing Chelex 100 (Bio-Rad) resin to chelate unbound metal ions. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to measure the concentration of divalent metals that remained in the protein sample (Fig 3).

Results

Before beginning metals testing, it quickly became apparent that a copurified protein contaminated the proteins composed of 9 amino acids. This protein was identified via mass spectrometry as HSP70 protein DnaK. With a ^{32}P ATP-binding assay, we compared a sample of purification tag-free 9C containing no detectable DnaK to a sample contaminated with DnaK (Fig. 5). We found that a sample of protein 9C with a trace amount of contaminant HSP70 DnaK showed 27% higher ATP-binding than a sample further purified to remove detectable contaminant (Student's t-test; p=0.007). To avoid interference in binding assays, our focus was shifted to the five amino acid library—specifically variant 5D.

Interestingly, during purification, 5D appeared to form a putative dimer. A higher molecular weight band was observed on an SDS-PAGE gel between analyses of the same sample (Fig. 4).





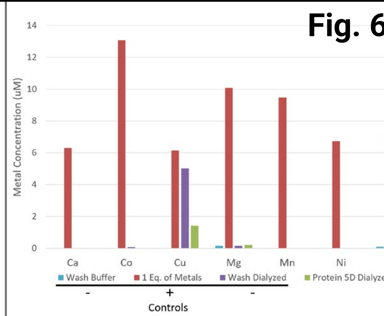


Figure 4. Putative dimer of protein 5D observed on LDS-PAGE gel Purification-tag-less 5D and MBP fusion 5D, NuPage 4-12% Bis-Tris. Samples loaded with NuPage LDS 4x loading dye and 5% beta-mercaptoethanol. A Precision Plus Protein Dual Xtra Standards (Bio-Rad) molecular weight (MW) ladder loaded in the rightmost lane.

Figure 5. Variant 9C's ATP-Binding is artificially boosted by contaminant DnaK Percent ATP bound calculated as the difference in measured radioactivity of the retentate and filtrate out of total retentate radioactivity in counts per minute. Each sample was run in duplicate with error bars indicating +/- standard error of the mean. Pairwise student's t-tests were performed for 9C+DnaK vs no protein, p=0.007; vs 9C, p=0.002; and no protein vs. 9C, p=0.059.

Figure 6. Inconclusive ICP-OES trace metal analysis of tag-free 5D Wash buffer (20 mM HEPES, 0.4 M KCl), a one equivalent (~3uM) metals solution, post-dialysis 5D incubated with metals (0.291 mM each), and post-dialysis wash buffer incubated with metals (0.291 mM each). Concentrations of metal measured were significantly variable in the ~3uM metals solution, copper levels appeared elevated in the dialyzed samples, and the negative control dialyzed wash buffer had elevated metal content.

Results cont.

Both bands were confirmed to be 5D via mass spectrometry. This led to the theory that 5D may have formed a denaturant-resistant dimer. We have also observed a negligible effect of LDS on 5D's retention factor. Since both are highly negatively charged at neutral pH, we expect minimal interaction of 5D with SDS/LDS detergents.

Variant 5D was purified as free protein and incubated with 6.6x equivalents of each metal for one hour. It was then dialyzed and submitted to ICP-OES analysis (Fig. 6). Unfortunately, the negative control for dialysis failed, leading to inconclusive results on metal binding. Several other anomalies include the uneven concentrations of divalent cations in the one equivalent positive control and the high amount of copper in both the experimental and negative control dialyzed samples.

Future Directions

- Repeat ICP-OES trace metal testing with new metals stock solutions, multiple sample replicates, and multiple rounds of dialysis for more stringent removal of metal ions
- Continue ICP-OES characterization of trace metals binding to the other protein variants, particularly those composed of five or nine amino acids
- Further investigate effect of metals on 5D's ATP binding affinity with individual metal treatments in a ^{32}P ATP-binding assay

Acknowledgements

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Citations:

[1]Newton, M. S., Morrone, D. J., Lee, K., & Seelig, B. (2019). Genetic Code Evolution Investigated through the Synthesis and Characterisation of Proteins from Reduced-Alphabet Libraries. *ChemBioChem*, *20*(6), 846–856. https://doi.org/10.1002/cbic.201800668

[2]Trifonov, E. N. (2004). The Triplet Code From First Principles. Journal of Biomolecular Structure and Dynamics, 22(1), 1–11. https://doi.org/10.1080/07391102.2004.10506975