Assay for Nitrite Reductase Activity in Engineered Metalloproteins

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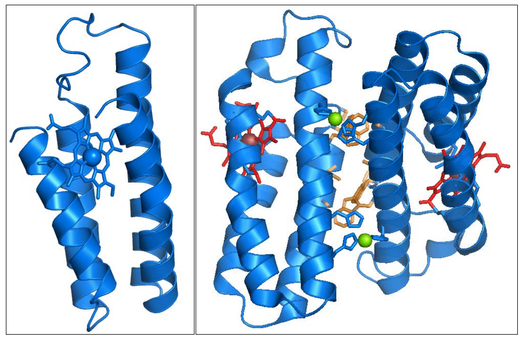
Abstract

Nature has developed many proteins that use metal ions generally as a scaffold for developing a stronger protein, or as an active site for substrate interactions. By creating proteins that use metal ions to develop interactions between proteins, not only are we able to exploit the strength and reversibility of coordination bonds, but we can also learn more about the structure and function of proteins with similar functions. In this study, we present an assay to create a nitrite reductase using RIDC-1, a mutated form of cytochrome *cb*$_{562}$ with ligand residues and hydrophobic interactions. Previous research has shown the similarities between Copper bonded to RIDC-1 and nitrate reductases. In this research, we present an assay to detect nitrite reductase activity in RIDC-1. Our study suggests that RIDC-1 is an active nitrite reductase and catalyzes this reduction with multiple turnovers. This result is a first step towards a better understanding of natural nitrite reductases and of better engineering metallic proteins.

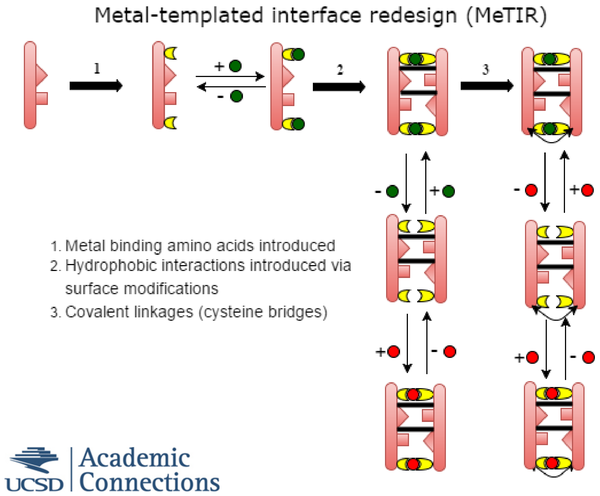
Background

Nature often spontaneously includes metallic ions in proteins to impart new structures and functions. For example, the metallo-beta–lactamase protein is an enzyme that breaks down antibiotics such as ampicillin, giving the bacteria antibiotic-resistance. Metallic ions form strong coordination bonds with proteins that are also reversible.

RIDC-1 is a protein engineered from cytochrome cb$_{562}$. Metal-templated interface redesign (MeTIR) facilitates this process by introducing metal coordinating residues and hydrophobic mutations. Our source of RIDC-1 came from *E. coli* that contains a plasmid encoding for RIDC-1.



The figure above demonstrates the transition from *cb*$_{562}$ RIDC-1. The figure below illustrates MeTIR



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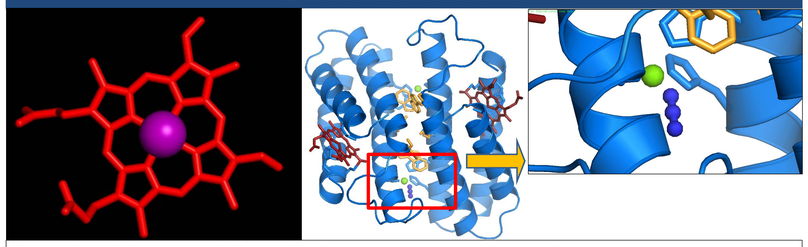
Structural Details



(from left to right): Heme, RIDC-1 with Copper, and the binding site of azide to copper

The heme cofactor in RIDC-1 facilitates purification because it makes the solution a distinctive crimson.

Previous research has demonstrated how RIDC-1 forms a tetramer with Zinc and a dimer with Copper where the former was coordinated four times and the latter was coordinated thrice. We reasoned that using Copper to form an RIDC-1 dimer leaves a binding site for a substrate, thus exhibiting catalytic behavior. Furthermore, RIDC-1 has been shown to bind with azide, a known inhibitor for nitrite reductase. Therefore, we reasoned that RIDC-1 could be used as a nitrite reductase

Nitrite Reductase

Copper based nitrite reductases catalyze the reduction of nitrite to nitric oxide, a gaseous radical with purposes ranging from neurotransmission to vasodilation.

Engineering enzymes that exhibit nitrite reductase behavior facilitates the understanding of not only alternative methods of reducing nitrite, but also of designing metalloproteins that exist in nature such that we can better understand their natural counterparts.

$$NO_2^- + 2H^+ + e^- \rightarrow NO + 2H_2O$$

$$[Cu(I) - RIDC1]_2 \qquad [Cu(II)RIDC1]_2$$

$$\frac{1}{2}\ ascorbate\ consumed$$

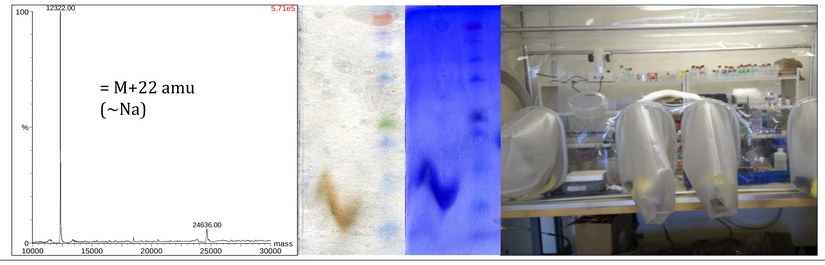
Procedure and Methodology

Prior to conducting any experiment, we had to produce and purify the protein.
- Express protein in *E. coli*
- Recover soluble protein in cell lysate
- Purify lysate via ion-exchange chromatography
- Obtain purified protein from size-exclusion chromatography
- Determine protein concentration via UV-Vis spectroscopy.

Assert Protein identity
- Determine protein mass via electrospray mass spectrometry to ensure protein identity.
- Sodium cation bonded to the protein
- Sample only contained RIDC-1

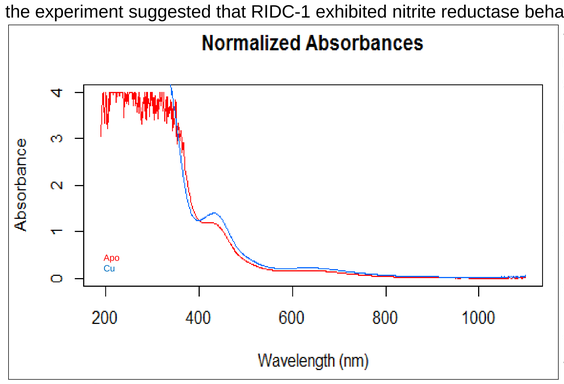
Assay for nitrite reductase:
- Anaerobic atmosphere required to prevent oxidation of NO (glove box)
- Sodium ascorbate used as sacrificial reductant to allow regeneration of RIDC-1
- Iron EDTA complex to trap produced NO (absorbs light at 432 nm).

(from left to right): Mass spectrum, Unstained gel, Coomassie stained gel, glovebox



Results

Our first execution of the experiment showed no signs of catalytic behavior. However, there were huge Oxygen leakages during the procedure, probably oxidizing all NO made. We repeated the experiment again being more careful to protect the isolated atmosphere. The second execution of the experiment suggested that RIDC-1 exhibited nitrite reductase behavior.



The graph to the left shows the normalized absorbance spectrums of the control (red) and the treatment with $CuCl_2$ (blue).

- Normalization required to account for volume differences.
- Peak at 432 nm strongly suggests catalytic activity
- Absorbance at 432 nm = 0.215
- $[Protein]_{final}$ = 116.8 μM

The RIDC-1 with Copper showed greater activity than RIDC-1. This difference in activity strongly suggests that RIDC-1 with Cu functions as a nitrite reductase.

Quantitative Enzymatic Data

Turnover number:

$$\frac{276\ \mu M}{117 \mu M} = 2.4$$

Conclusions

Our hypothesis for the experiment was that the dimerized protein coordinated to Copper would be able to serve as a nitrite reductase. Our results have given evidence supporting our assertion.

There were some minor errors in the procedure that we corrected with calculations or by redoing the experiment. In the first setup of the experiment, the anaerobic atmosphere was exposed to oxygen when the glovebox leaked through the gloves. We reasoned that this oxidized the reagents, making the product undetectable. In the second run of the experiment, the volumes of the control and treatment were different. We amended this by normalizing the data. Although this theoretically fixes the values, the experiment must be repeated to verify this.

Future developments are not limited to better understanding nitrite reductases but metalloproteins engineering and function in nature. Additional research can be done on other RIDC-1 proteins to determine enzymatic properties. By observing engineered metalloproteins, we can compare their structures to natural ones to better understand the relation between structure and function in natural proteins along with the evolutionary subtleties that allow these proteins to function in such an optimized state. This study additionally reinforces the growing method of combining both repurposed protein folds with redesigning proteins from scratch as a useful method of not only engineering proteins but discovering their analogues in nature.

References

1. J.B. Bailey, et al., Metal-Directed Design of Supramolecular Protein Assemblies, Methods in Enzymology, Academic Press.
2. Salgado, Eric N., et al. "Metal Templated Design of Protein Interfaces."*Proceedings of the National Academy of Sciences* 109.52 (2010): *Metal templated design of protein interfaces*. 23 Dec. 2009. Web.

PDB codes used:
2BC5, 3HNI

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