The Challenge of Finding Where SOD Gets Zinc: Identifying Novel Techniques to Recombinantly Express Metallothionein

University Honors College and Bioresource Research Thesis
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Amyotrophic Lateral Sclerosis (ALS)

5,600 Americans Diagnosed Every Year With ALS

20% of patients live past 5 years and 10% live past 10 years

[Doctor Tipster, 2011]
Introduction

- Amyotrophic Lateral Sclerosis
  - Mutations in Cu,Zn-Superoxide Dismutase
- Where does SOD get Zinc?
  - Metallothionein
Superoxide Dismutase (SOD) and Familial ALS (fALS)

- Daniel Rosen
  - Correlation between mutant SOD and fALS

- Mutated SOD
  - 130 mutations discovered

- fALS vs. sALS
  - Clinically similar
  - Mutant SOD Accounts for 2% of Total Cases

Mutated SOD → Controversial Mechanism of Disease → Familial ALS Onset
Superoxide Dismutase (SOD) Structure

Homodimer
- Cys57-Cys146 disulfide bond

Unusual Stability
- Copper and zinc are coordinated with specific amino acids
- Copper and zinc must be bound for proper SOD functioning

[Roberts. Journal of Molecular Biology. 2007]
Superoxide Dismutase Function

\[ \text{O}_2^- + \text{Cu(II)ZnSOD} \rightarrow \text{O}_2 + \text{Cu(I)ZnSOD} \]  (1)

\[ \text{O}_2^- + \text{Cu(I)ZnSOD} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{Cu(II)ZnSOD} \]  (2)

[Hart. Biochemistry. 1999]

[Roberts. Journal of Molecular Biology. 2007]
Zinc Deficient SOD and fALS

[Roberts. Journal of Molecular Biology. 2007]
Zinc Deficient SOD Toxic Gain of Function

**Nitric Oxide (NO)**
- Created by Nitric Oxide Synthase (NOS)
- $\text{O}_2^-$ reacts 3 times faster with NO than with WT,SOD

**Peroxynitrite (ONOO-)**
- Formed from NO and $\text{O}_2^-$

[SOD-$\text{Cu}^{2+}$ + ONOO$^-$ $\rightarrow$ SOD-$\text{CuO}$...NO$_2^+$]

[SOD-$\text{CuO}$---NO$_2^+$ + H---Tyr $\rightarrow$ SOD-$\text{Cu}^{2+}$ + HO$^-$ + NO$_2$-Tyr]

Proposed Mechanism for ALS

1. Mutant SOD
2. Zinc Deficient SOD
3. SOD Toxic Gain of Function
4. Nitration of HSP90
5. Motor Neuron Cell Programmed Death
6. ALS Onset
The Mystery of Where SOD Receives Zinc
Metallothionein (MT)

Margoshes and Vallee discovered a cadmium binding protein in horse liver (1957).

Vallee and Kägi called the cadmium binding protein Metallothionein and note its ability to bind zinc (1961).

Maret and Vallee proposed a novel cell functioning of MT not as an anti-oxidant protein, but as a zinc concentration regulator (1998).

[Kojima, 1976]
Metallothionein Structure

**Thionein**
- Apo (non metal) form of MT
- No known tertiary structure
- Molecular Weight is 6-7 kDa

**2 Domains**
- $\alpha$ Domain binds 3 zinc atoms
- $\beta$ Domain binds 4 zinc atoms
Metallothionein Cellular Function

[Graph showing the relationship between time and absorbance and Zn atoms released.]

[Diagram illustrating the metallothionein cycle and its function.]


Theoretical Connection of MT and SOD

- Reduced Form of Thionein Binds Zinc
- Zinc Bound Metallothionein Interacts With an Oxidizing Agent
- Oxidizing Agent Causes Release of Zinc to Apoenzyme
- SOD Could Be A Potential Apoenzyme Target
## Research Objectives

<table>
<thead>
<tr>
<th>Create Recombinant MT Gene</th>
<th>Express MT with BL21(DE3) pLysS System</th>
<th>Express MT with Shuffle T7 System</th>
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</thead>
</table>
| • Insert MT gene into pTYB11 plasmid | • Insert MTpTYB11 plasmid into competent BL21(DE3) pLysS cells  
• Induce with IPTG | • Insert MTpTYB11 plasmid into competent Shuffle T7 cells  
• Induce with IPTG |
Methods – Create Recombinant MT Gene

- \textit{Create Recombinant MT Gene}

- \textit{pTYB11 Plasmid}

- \textit{EcoRI}

- \textit{SapI}

- Intein Protein Coding Region (Purple)
Results – Create Recombinant MT Gene

Indirect Verification of Correct Insertion
1% Agarose Gel
Results – Create Recombinant MT Gene

pTYB11 DNA Sequencing

- pTYB11 Sequence
- CGRB Sequence
Results – Create Recombinant MT Gene

MT DNA Sequencing

- MT Sequence

- CGRB Sequence
Methods - Express MT with BL21(DE3) pLysS System
Results - Express MT with BL21(DE3) pLysS System

SDS Gel – BL21 Expression

~63 kDa
Overcoming Challenges

<table>
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<th>Express MT with Shuffle T7 System</th>
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<tbody>
<tr>
<td>• T7 RNA Polymerase</td>
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</tr>
<tr>
<td>• T7 Lysozyme</td>
<td>• T7 Lysozyme deficient</td>
</tr>
<tr>
<td>• OmpT protease</td>
<td>• OmpT protease deficient</td>
</tr>
<tr>
<td>• Thioredoxin</td>
<td>• Lon protease deficient</td>
</tr>
<tr>
<td>• Glutaredoxin</td>
<td>• Glutaredoxin and thioredoxin deficient</td>
</tr>
<tr>
<td>• Lon protease</td>
<td>• DsbC chaperone protein</td>
</tr>
</tbody>
</table>
Methods – Express MT with Shuffle T7
Results – Express MT with Shuffle T7

BL21 Expression

Shuffle T7 Expression

~63 kDa
Results – Express MT with Shuffle T7

Li-Cor Western Blot
Successful creation of recombinant plasmid!! Verified by DNA sequencing.

Successful expression of intein protein with Shuffle T7. Verified by SDS gel.

Unsuccessful expression in BL21 System. Verified by SDS gel.

Inconclusive expression of MT using Shuffle T7. Analyzed using Western Blot.
Future Plans – In A Perfect World

Apo SOD + Zinc Bound MT = Zinc Bound SOD?

Cu,-SOD + Zinc Bound MT = Cu,Zn SOD?

Cu,Zn SOD + Apo Thionein = Zinc Bound MT
Implications of MT Research

- Increased Understanding of ALS
- Characterization of SOD
  Postranslational Metal Binding
  Sequence of Events
- Process to Purify Metallothionein
Acknowledgements

• **Beckman Lab**
  - Principle Investigator – Dr. Joseph Beckman
  - Nathan Lopez
  - Pamela Beilby
  - Ed Labut
  - Jared Williams
  - Nick Rosson

• **Environmental and Molecular Toxicology Department**
  - Secondary Mentor – Dr. Daniel Sudakin

• **Howard Hughes Medical Institute**
  - Kevin Ahern

• **Environmental Health Sciences Center**

• **Bioresource Research**
  - Department Head – Dr. Kate Field
  - Advisor – Wanda Crannell

• **Friends and Family**
  - Russ and Mary West
  - Reva West, J.D. and Audrey Cant, Jamie and Tony Haddock
  - Alex Sutherland
  - Fraternity of Phi Gamma Delta
Thank You

Are there any questions?