



FINAL REPORT

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GRANT TITLE: Molecular Approaches to Optical Biosensors

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OBJECTIVE: The overall objective is to develop and use molecular biology methods for optimizing protein molecules as biomaterials, including their use in field-deployable optical biosensors. Our immediate objective is to improve the metal ion detection limit and to vary the metal ion specificity of a carbonic anhydrase-based fiber optic metal ion biosensor.

APPROACH: Use molecular biology methodologies to optimize a carbonic anhydrase-based metal ion biosensor, including: improving the metal ion detection limit, varying the metal ion specificity; optimizing reagentless signal transduction methods and developing enhanced immobilization methods.

ACCOMPLISHMENTS: *Reagentless Biosensor*: We have prepared CA variants containing a novel cysteine residues at various positions on the surface of the protein, including the active site cavity. We have labeled this cysteine with a fluorophore and demonstrated that these proteins can be used for reagentless determination of picomolar levels of zinc and copper from changes in: (1) the phase shift of ABD-labeled N67C CA; and (2) the fluorescence anisotropy of ABD-labeled H64C CA. The sensitivity of these fluorimetric zinc assays is significantly lower than previous determinations.

We then prepared CA variants with multiple amino acid substitutions for use in the development of metal ion biosensors. In particular, we made and characterized > 10 variants containing double and triple substitutions which: (1) enhance labeling with fluorophores (removal of native cysteine and insertion of novel cysteine at position 67, 198 or 131); and (2) increase kinetics of metal binding (substitutions at E117). Our collaborator, Richard Thompson, has demonstrated that sensors using these proteins as transducer elements respond more rapidly to metals in solutions while retaining similar metal ion sensitivity.

*Development of Immobilization Tags*: We have used PCR mutagenesis to insert unique restriction sites at the end of the CA gene for ease of insertion into commercially available vectors that will add "tags" onto the protein (*i.e.* biotin,

streptavidin or S peptide) for oriented immobilization onto a surface. We also have been developing a unique thiol specific tag which can be covalently attached in an oriented manner to a thiol-reactive self-assembled monolayer on a glass surface.

*Metal Specificity:* Substitutions in the conserved aromatic residues F93, F95, and W97 underneath the zinc polyhedron in carbonic anhydrase significantly alter the metal specificity of CAII by: decreasing zinc and cobalt affinity, increasing copper affinity and having little effect on the nickel affinity. Furthermore, these changes correlate with the volume of the side chains at these positions, allowing the design of metal specificity. The T93/S95/V97 variant binds Cu(II) with sub-femtomolar affinity (~200-fold tighter than wild-type) and the Cu/Zn and Co/Zn specificity increases 20,000-fold and 8-fold, respectively. X-ray crystallographic studies of these proteins (with Dr. David Christianson) demonstrate that these alterations are due to structural rearrangements in the apo-enzyme that alter the pre-organization of the metal polyhedron. Measurements of the metal affinity of denatured and native CAII also demonstrate that high zinc affinity Zn/Cu specificity is attained by pre-organization of the metal polyhedron in the apo-enzyme.

We also determined the metal affinity [Zn(II), Cu(II), Co(II), Cd(II), Mn(II) and Ni(II)] of a set of CAII variants with Asp, Glu, Gln and Asn substituted for one of the His residues that coordinate zinc in wild-type. In general, these substitutions significantly decrease the affinity of zinc and alter the metal specificity of the variants. For example, the H94D substitution decreases the copper and zinc affinity with little effect on the affinity of other metals; therefore the metal specificity switches from Cu>Zn>Cd>Ni>Co>Mn for wild-type CA to Cu>Cd>Zn~Ni>Mn for H94D CA. Additionally, the Cu/Zn affinity ratio varies from 1 for H119Q CA to 10 for wild-type CA to  $6 \times 10^4$  for H119N CA. These alterations suggest that the positioning of the protein and water ligands to achieve optimal metal geometry plays a large role in determining metal ion specificity in CA.

Finally, we measured the Zn(II)/Cd(II) metal specificity of a set of CAII variants with Cys substituted for one of the His residues that coordinate zinc in wild-type. We proposed that these variants would have increased specificity for the more thiophilic metal, Cd(II), compared to Zn(II). These mutations decrease both the affinity for zinc and cadmium and, unexpectedly, have little affect of the Zn/Cd ratio.

*Phage display:* We used sulfonamide affinity chromatography to select CA-phage with high zinc affinity out of a library of variants randomized at positions 93, 95 and 97. The zinc affinity of these selected variants is comparable to wild-type (1-20 pM). Furthermore, we have demonstrated that both the zinc dissociation constant and the dissociation rate constant correlate with the total volume of the amino acids

at these three positions. This correlation allows the design of variants with predictable zinc binding properties. Finally, we prepared larger CA-phage libraries with alterations in 12 amino acids in CA to screen for variants with altered metal ion specificity. We have carried out multiple selection rounds for variants with altered Zn/Cu affinity ratios using sulfonamide chromatography, have sequenced several of these variants, and are characterizing their properties.

CONCLUSIONS: We are able to use molecular biology techniques to optimize the properties of carbonic anhydrase for a field-deployable metal ion biosensor. In particular, the zinc/copper specificity can be tuned by making mutations in the hydrophobic amino acids beneath the zinc binding site. Furthermore, the protein can be labeled with fluorophores at single engineered cysteine to enhance the sensitivity of zinc sensing.

SIGNIFICANCE: Single cysteine variants have been used to increase the sensitivity of zinc fluorimetric assays. Furthermore, we have prepared CA variants that alter the half-time for metal equilibration, the zinc dissociation constant, and the metal ion specificity. These variants will be useful in the development of sensor arrays to determine the concentration of multiple metal ions simultaneously in wastewater and the ocean.

PATENT INFORMATION:

Thompson, R. B., Felliccia, V. L., Maliwal, B. P. and Fierke, C. A. (2001) Photoluminescent Sensors of Chemical Analytes, U.S. Patent Number 6,197,258 B1, UM Tech ID No. 1458RT.

AWARD INFORMATION: NONE

PUBLICATIONS AND ABSTRACTS (for total period of the grant):

1. Christianson, D. W. and C. A. Fierke. 1996. Carbonic Anhydrase: Evolution of the Zinc Binding Site by Nature and by Design. *Acct. Chem. Res.* 29: 331-339.
2. Thompson, R. B., Z. Ge, M. Patchan, C.-C. Huang, and C. A. Fierke. 1996. Fiber Optic Biosensor for Co(II) and Cu(II) Based on Fluorescence Energy Transfer with an Enzyme Transducer. *Biosensors and Bioelectronics* 11: 557-564.
3. Lesburg, C. A., C.-c. Huang, D. W. Christianson, and C. A. Fierke. 1997. Histidine→Carboxamide Ligand Substitutions in the Zinc Binding Site of Carbonic Anhydrase II Alter Metal Coordination Geometry but Retain Catalytic Activity. *Biochemistry* 36:15780-15791.