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13. ABSTRACT *(Maximum 200 words)*
This ASSERT proposal supports graduate assistants working on projects related to the parent grant, Molecular Approaches to Optical Biosensors, which proposes to optimize a carbonic anhydrase-based fluorescence biosensor for detecting metals. Our current goals were to use mutagenesis to prepare carbonic anhydrase variants with: (1) altered metal ion specificity by varying the size, geometry and chemical structure of direct and indirect metal ligands; and (2) increased protein stability by insertion of disulfide bonds. The metal ion specificity of CA variants with alterations in the histidines that coordinate the metal ion is altered significantly compared to wild-type. For example, the Cu/Zn affinity ratio varies from 1 for H119Q CAII to 10 for wild-type CAII to 6×10^4 for H119N CAII. These variants should be useful for either preparing copper-specific sensors or for measuring multiple metal ions simultaneously using a CA-based biosensor. Furthermore, analysis of double cysteine variants indicates that the PAIRWISE potential is a good predictor of the strength of a novel disulfide bond, however, the destabilizing effects of cysteine substitutions on the native state and the stabilization of compact non-native states by the disulfide can override any stabilizing effect of the crosslink.

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FINAL PROGRESS REPORT

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GRANT TITLE: Biosensor Design: Combinatorial and Rational Approaches

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OBJECTIVE: This ASSERT proposal supports graduate assistants working on projects related to the parent grant, Molecular Approaches to Optical Biosensors, which proposes to optimize a carbonic anhydrase-based fluorescence biosensor for detecting metals. Our current goals are to develop carbonic anhydrase variants with altered metal ion specificity and increased protein stability.

APPROACH: We are using mutagenesis to prepare variants with: (1) altered metal ion specificity by varying the size, geometry and chemical structure of direct and indirect metal ligands; and (2) increased protein stability by insertion of disulfide bonds. We then characterize the metal affinity, metal specificity, and stability of these variants.

ACCOMPLISHMENTS:

Metal ion specificity: We have developed colorimetric and fluorometric assays to rapidly assay a variety of metal ion concentrations. We then used equilibrium dialysis combined with these assays to determine the concentration of bound metals in order to measure the metal ion 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 histidines (either His-94 or His-119) that coordinate zinc in wild-type CAII. In general, these substitutions significantly decrease the affinity of zinc, indicating that the metal-ligand coordination is suboptimal. However, these variants with decreased metal affinity can be used in the CAII-based biosensor to expand the dynamic range of zinc concentrations measureable.

Furthermore, these data demonstrate that the metal specificity of wild-type CA follows the "Irving-Williams" series except that zinc affinity is higher relative to copper than is observed in most small molecule chelators. In many cases the metal ion specificity of these CA variants is altered significantly compared to wild-type. For example, H94D substitution decreases 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-

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type CAII to Cu>Cd>Zn~Ni>Mn for H94D CAII. Additionally, the Cu/Zn affinity ratio varies from 1 for H119Q CAII to 10 for wild-type CAII to 6×10^4 for H119N CAII. 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 CAII. To date, we have obtained no evidence indicating that the polarizability of the coordinating atom influences the metal specificity of the active site of CA. These variants should be useful for either preparing copper-specific sensors or for measuring multiple metal ions simultaneously using a CA-based biosensor.

Protein stability: Rationally-designed disulfide bonds offer an attractive means of stabilizing the native fold of a protein. An analysis of the pairwise side chain packing geometries of cysteine residues observed in high-resolution protein crystal structures indicates that cysteine pairs have pronounced orientational preferences due to the geometric constraints of disulfide bond formation. These observations were used to generate a potential function to evaluate models for novel disulfide bonds in human carbonic anhydrase II (HCAII). Three double-cysteine variants of HCAII were purified and characterized. The S29C/S197C variant is readily reduced by glutathione, consistent with the PAIRWISE prediction that the native backbone structure was not consistent with disulfide bond formation even though the C_b atoms in these residues should be close enough to interact. The A38C/A258C and L60C/S173C variants are more resistant to reduction; no detectable concentration of the thiol forms of these proteins could be found at the [GSH]/[GSSG] ratios studied. Reduction of these variants was possible using DTT, which is a stronger reducing agent. The A38C/A258C variant was fully reduced at all ratios studied, indicating that the redox potential for this disulfide is intermediate between gluathione and DTT. The equilibrium constant for reduction (K_{red}) of the L60C/S173C variant by DTT was determined to be 0.6, comparable to the K_{red} observed for the 14-38 disulfide bond in bovine pancreatic trypsin inhibitor and the effective concentrations of their thiol groups were determined by titrations with glutathione and dithiothreitol. These analyses indicate that the PAIRWISE potential is a good predictor of the strength of the disulfide bond itself, but the overall structural and thermodynamic effects on the protein are complicated by additional factors. In particular, the destabilizing effects of cysteine substitutions on the native state and the stabilization of compact non-native states by the disulfide can override any stabilizing effect of the crosslink.

SIGNIFICANCE: We have prepared CA variants that alter both the zinc dissociation constant, and the metal ion specificity. Furthermore, we have successfully prepared a CA variant with a disulfide bond linking residues C60 - C173

that is folded and is a start toward producing CA variants with increased stability. These variants will be useful in the development of sensor arrays to determine the concentration of multiple metal ions simultaneously.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1) Thompson, R. B., Maliwal, B. P., Feliccia, V. L., Fierke, C. A. and McCall, K. M. (1998) Determination of picomolar concentrations of metal ions using fluorescence anisotropy: biosensing with a "reagentless" enzyme transducer, *Anal. Chem* **70**, 4717-4723.

2) Thompson, R. B., Ge, Z., Patchan, M., Fierke, C. A., McCall, K. A., Elbaum, D. and Christianson, D. W. (1996) Determination of Multiple Analytes using a Fiber Optic Biosensor Based on Fluorescence Energy Transfer, *Progress in Biomedical Optics: Proceedings of Ultrasensitive Biochemical Diagnostics* , vol. 2680, (A.Katzir, ed.), SPIE, 47-56.

3) McCall, K. A., Huang, C.-c. and Fierke, C. A. (1999) Function and Mechanism of Zinc Metalloenzymes, *J. Nutr.*, in press.

4) Burton, R. E., Fierke, C. A. and Oas, T. G., Novel disulfide engineering in human carbonic anhydrase II using the PAIRWISE side-chain geometry database, in preparation for submission to *Protein Science*.

5) McCall, K. A. and Fierke, C. A. Metal ion specificity and avidity in carbonic anhydrase variants, in preparation for submission to *Biochemistry*.