Characterization of Copper Binding to Riboflavin Binding Protein

Rebecca Shaw, Kristen Wasiukanis, Professor Sheila Smith, Professor Marilee Benore-Parsons
Department of Natural Sciences, University of Michigan- Dearborn

Abstract

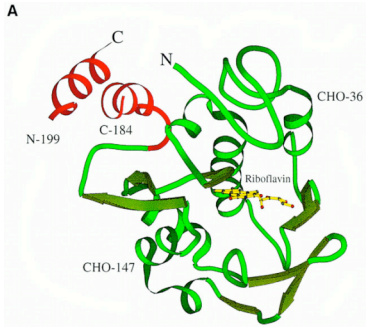
Riboflavin Binding Protein (RBP) is a small monomeric protein purified from chicken eggs; its known function is in the transport of the essential nutrient riboflavin into the egg. Recently, we have shown that RBP binds copper in a 1:1 molar ratio. This binding interaction suggests a possible additional role for RBP in the transport and storage of copper in avian embryo.

Why use Chicken eggs?

- Large quantities of fetal nourishment are transferred into eggs.
- By testing eggs we do not have to test live animals.
- We can easily purify the protein.
- Possible designer proteins can be synthesized by using eggs.

Structure of RBP

- 3 isoforms- yolk, white, serum
- All are glyco-phosphoproteins
- All contain 9 disulfide bonds
- All bind riboflavin
- Variations in cleavage
- Variations in glycosylation
- Variations in phosphorylation
- Sequence similarity to folate binding protein family

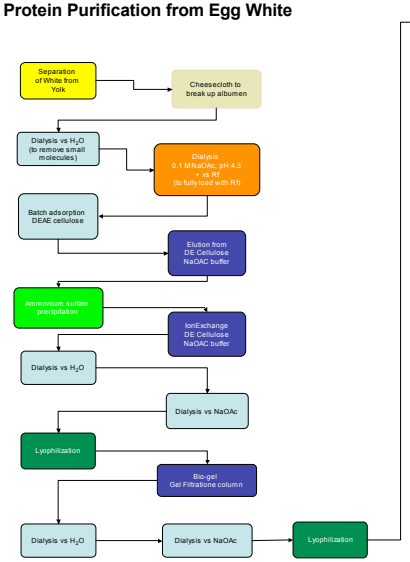


Crystal structure of chicken riboflavin-binding protein
Hugo L. Monaco
Copyright © 1997 by the European Molecular Biology Organization

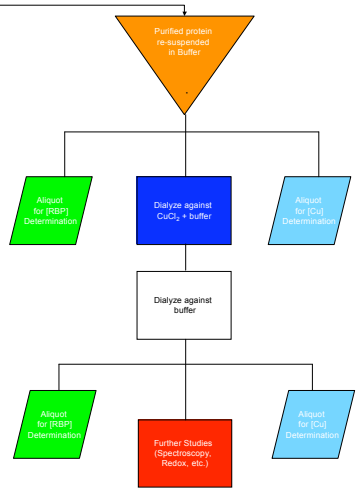
Cu Binding Under Dialysis Conditions

Protein is re-suspended in buffer and dialysed against buffer solution containing excess Cu^{2+}, followed by dialysis against buffer solution to remove any copper that is not tightly bound to the protein.

Protein Purification from Egg White



- Egg white is isolated and forced through cheesecloth
- Dialysis against buffer (pH 4.3, Na acetate)
- Batch absorption to DE cellulose and column elution using sodium chloride
- Differential precipitation using ammonium sulfate
- DEAE ion exchange chromatography
- Gel filtration chromatography
- SDS-PAGE and spec analysis



Determination of [RBP]

[RBP] is determined by Beer's Law (A= ε cl) using the extinction coefficient (Molar Absorptivity, ε) at 280 nm.

Determination of Copper Bound to RBP

A calibration curve is created using a range of concentrations of a commercial copper standard solution.

Measurement of the unknown sample (the dialyzed protein, ★) gives the concentration of copper in the solution.

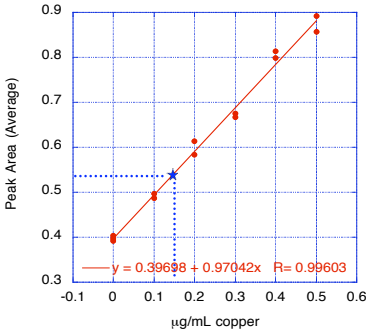
In a separate experiment, the protein concentration of the same solution is determined by use of an extinction coefficient and Beer's Law, with a correction for the absorbance of bound riboflavin.

Combining these two pieces of data, we arrive at a ratio of copper to protein.

On average, we see copper binding in an approximately 1:1 molar ratio.

Copper has been detected at very low levels in the protein as purified. We have shown that copper bound to the protein under dialysis conditions does not survive treatment with the pH 4.3 acetate buffer used in purification.

Cu Analysis by Graphite Furnace AA Calibration Curve



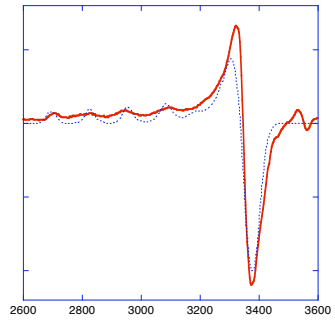
Unknown Data

0.158 µgCu/mL solution

0.249 mM Copper

0.290 mM protein solution

0.86 mol Cu/mol RBP

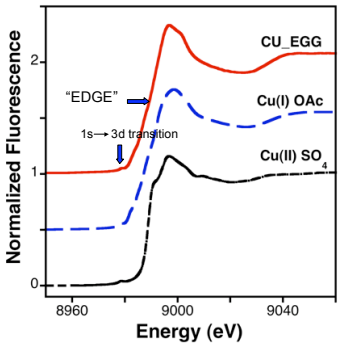


The protein (dialyzed against copper) was examined in frozen solution (10 K) by electron paramagnetic resonance. The spectrum shows a single well-ordered Type II copper site. The site is axial with

$g_{\perp} = 2.065$
$(A_{\perp}=10\times10^{-4}\ cm^{-1})$

$g_{||}=2.39$
$(A_{||}=142\times10^{-4}\ cm^{-1})$.

Comparison to other reported copper complexes suggests a tetra-oxygenate environment for the copper in the protein.



The protein was examined by X-ray absorption spectroscopy by our collaborators at Wayne State University (Stemmler lab).

Edge energy shows mixture of copper (I) and copper (II). The small feature at ~8980 eV is the 1s→3d transition, indicating copper (II), but the edge itself looks more like that of Cu(I).

EXAFS (X-ray Absorption Fine Structure) not shown) indicates oxygen in first coordination sphere. There may also be a Nitrogen (Histidine).

*This work is supported by a grant from the Office of the Vice Provost for Research of UM and by the UM-Dearborn Office of Sponsored Research Programs through several Undergraduate Research Fellowships. Much of this work has been published in the Journal of Inorganic Biochemistry, (*J.Inorg.Biochem., 100 (**2006**), 1730-1733.*) Special Thanks to the McCracken Lab and The Michigan Center for Structural Biology at Michigan State University for the use of the EPR instrument and to the Stemmler Lab at Wayne State University for XAS studies.*