Investigation on Enzyme Inhibition Mechanism

Lauren Ebersol, Robert Powers, PhD

Department of Forensic Science, Henry C. Lee College of Criminal Justice and Forensic Science

INTRODUCTION

Transferrin is the primary iron transport protein in the body. It's synthesis involves, in addition to the underlying 679-amino acid core, a "glycosylation" process wherein multiple sialyl (N-acetylneuraminic acid; Neu5Ac) residues are added to the protein. Alcoholics, or habitual drinkers characteristically exhibit some degree of diminished efficiency in that process, and "carbohydrate deficient transferrin" (CDT) has been proposed as a marker for chronic alcoholism. CDT is currently being used clinically as an indicator of compliance, or lack thereof, with alcohol-treatment programs in Europe. CDT levels are being considered for their possible applicability in various ways within the criminal justice system of the United States as well.

Despite the apparent correlation between carbohydrate deficient transferrin and alcoholism, there has been no mechanism proposed for how this occurs on a molecular level. We hypothesize that ethanol acts as a direct, competitive inhibitor of the enzymatic system responsible for adding sialyl residues to the transferrin molecule, the "sialyltransferases". Sialic acid is a cyclic nine-carbon sugar analog. When attached to cytidine 5'-triphosphate (CTP), sialic acid yields the activated substrate CMP-Neu5Ac, which may then be taken up in the Golgi apparatus as a substrate for sialyltransferase activity, transferring the sialyl residue to the unconjugated protein.

Enzyme inhibition can be determined in the laboratory, including the mechanism by which it occurs. There are three different types of inhibition: competitive, non-competitive and uncompetitive. In the "competitive" case, the inhibitor competes with the normal substrate of the enzyme for the active, or "binding site" of the enzyme. The presence of a competitive inhibitor can be inferred experimentally by observing a change in the ability of the enzyme to bind the substrate (Km) in the presence of the supposed inhibitor. Non-competitive inhibitors act on the enzyme themselves, affecting the ability of the molecule to catalyze the specific reaction, without affecting substrate binding. Experimentally, this can be observed as a change in the maximal rate of reaction of the enzyme (Vmax). Finally, uncompetitive inhibition occurs as the inhibitor interacts with the enzyme-substrate complex, resulting experimentally, in changes in both substrate binding and maximal velocity (Km & Vmax). The nature of inhibitor action can be determined using a plot of reaction rates as substrate concentration is varied, allowing evaluation of the reaction kinetic variables.

Initial experiments on enzyme kinetics and inhibition, including substrate-velocity experiments, and calculations of Km, Vmax and Ki in a competitive-inhibitory system were carried out using rat cytosolic alcohol dehydrogenase, and pyrazole as an inhibitor.

Because of difficulties anticipated in working with transferrin, and transferrin sialyltransferase, the model system chosen was a rabbit cytosolic lactose sialyltransferase. In this system, the sialyltransferase catalyzes the sialylation of lactose, yielding sialyllactose, in a manner analogous the sialylation of transferrin.

METHOD & MATERIALS

Incubation solutions were created to be a total of 40µL. A total of seven solutions were made: rabbit serum (Sigma) and Tris-HCl buffer; rabbit serum, lactose (Sigma), and Tris-HCl buffer; rabbit serum, CMP-Neu5Ac (Sigma), and Tris-HCl buffer; rabbit serum, lactose, and CMP-Neu5Ac. Tris-HCl buffer was prepared by dissolving Trizma base salt (Sigma) in deionized water (obtained using Millipore System), then using and adjusting the pH using AB15 pH meter, hydrochloric acid (Sigma-Aldrich), and sodium hydroxide (Fisher) to pH 6.75, with the final concentration being 100 millimolar (mM). Tris-HCl buffer was used to correct the volume to 40µL for each sample. The lactose solution was prepared using D-lactose monohydrate (Fisher) and 100mM Tris-HCl buffer, and the concentration created was 2.40mM. Lactose added to the samples was 15µL. CMP-Neu5Ac solution was prepared from CMP-Neu5Ac powder and 100mM Tris-HCl buffer, with a final concentration of 2.40mM. CMP-Neu5Ac added to the samples was 15µL. Rabbit serum was used directly, and the amount added was 10µL. Each assay solution prepared was either stopped at time zero with 200µL of 0.100M acetic acid (VWR Scientific), or allowed to incubate at 40 degrees Celsius in a water bath for one hour, then killed using 200µL of acetic acid. Incubations were done using HotShaker water bath. All solutions were stored at 4 degrees Celsius after incubations.

Analysis of all incubations was performed on a high performance liquid chromatography/triple quadrupole mass spectrometry instrument with a hydrophilic interaction liquid chromatography (HILIC) column (Luna 3u HILIC 200A 50x3.3mm, Phenomex). Further analysis of lactose and a 3'-sialyllactose standard (Sigma) was performed using Agilent 1100 series high performance liquid chromatography instrument.

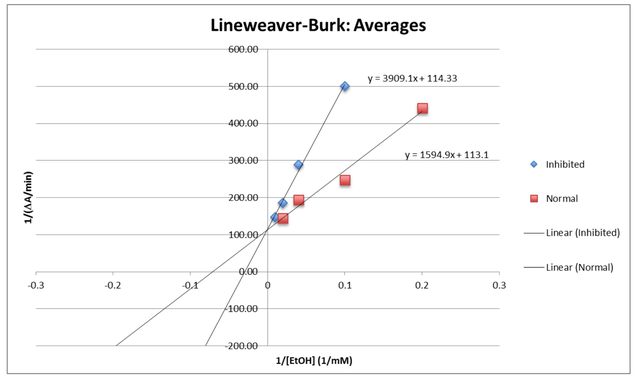
Shown above is the Agilent 1100 Series high-performance liquid chromatography instrument used for analysis

Shown above is the high performance liquid chromatography/triple quadrupole mass spectrometry instrument used at the Connecticut State Crime Lab for analysis.

RESULTS

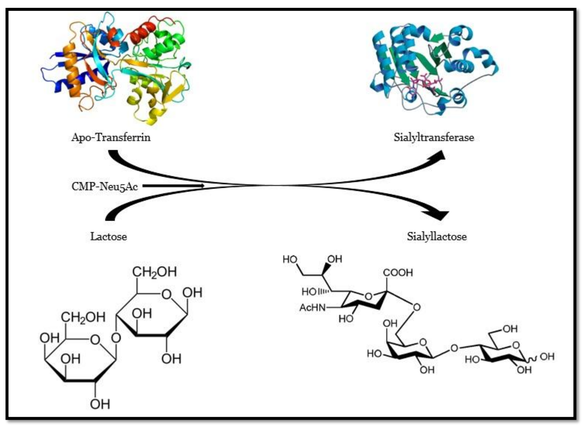
Preliminary experiments using alcohol dehydrogenase as a basis for demonstrating competitive inhibition via substrate-velocity and Lineweaver-burke plots using rat cytosol were straightforward, and without complication. The analogous experiments with cytosolic sialyl transferase are awaiting final analysis because of time and instrument issues with the Triple-Quadrupole Mass Spectrometer.

We have as yet failed to establish a method for the analysis of 3'-sialyllactose using high performance liquid chromatography. We are currently utilizing a gradient mobile phase system, transitioning from 10% acetonitrile to 50% acetonitrile in 10 mM ammonium acetate at 0.5 mL/min. Diode array detector was set at 250 nm, with 50 ul injection volume.



Shown above is an example of competitive inhibition, derived from the Lineweaver Burke plot

Shown below is a figure showing the similarities between sialylation of lactose and transferrin



DISCUSSION

Evaluation of an enzyme assay and determination of a classic Ki for pyrazole/alcohol dehydrogenase, performed as a training exercise for the evaluation of inhibition of sialyltransferase was achieved without complication.

Development of a direct analytical method for sialyllactose by HPLC in incubation samples was not resolved by the completion of the project. (The analytical standard of sialyllactose only became available in August). Nevertheless, the remaining analytical issues are expected to be resolved, allowing direct analysis of incubation samples by HPLC. Similarly, we are awaiting results from LC-MSMS instrumentation. While routine analysis of incubation samples is expected to be performed on HPLC, the combination of HPLC and triple quadrupole mass spectrometry (LC-MSMS) allows for a higher degree of sensitivity, which may be important during the process of assay optimization.

Further analysis following this project will continue to evaluate ethanol as an inhibitor on the sialylation of lactose (as a model for transferrin sialylation). This project was an important stepping stone for these further analyses. A link between carbohydrate deficient transferrin and ethanol has been establish and accepted, yet no mechanism for this enzymatic inhibition has been proposed. There are not many other components of this reaction that could cause the inhibition, so ethanol is an obvious first step. Although present in low quantities, ethanol has an effect on all proteins, and it is possible that this specific protein is especially sensitive to ethanol. Further analysis is especially important because it difficult to justify and accept a parameter for being a valid indicator, when there is no mechanism proposed or accepted for that action.

REFERENCES

Devlin, T. M. (Ed.). (2002). Textbook of biochemistry with clinical correlations (5th ed.). New York, NY: Wiley-Liss.

Ghosh, P., Okoh, C., Liu, Q.-H., & Lakshman, M. R. (1993). Effects of chronic ethanol on enzymes regulating sialylation and desialylation on transferrin in rats. Alcoholism: Clinical and Experiment Research, 17(3), 576-579.

Jewett, S. L. (n.d.). Inhibition of enzyme activity [PowerPoint slides]. Retrieved July 10, 2015, from http://www.csun.edu/~hcchm001/5enzyme.pdf

Malagolini, N., Dall'Olio, F., Serafini-Cessi, F., & Cessi, C. (1989). Effect of acute and chronic ethanol administration on rat liver alpha-2,6-sialyltransferase activity responsible for sialylation of serum transferrin. Alcoholism: Clinical and Experimental Research, 13(5), 649-653.

NEUROtiker. (n.d.). *Lactose* [Image]. Retrieved from, http://biobook.nerinxhs.org/ bb/cells/biochemistry.htm

Protein Data Bank. (n.d.). *Transferrin* [Image]. Retrieved from https://en.wikipedia.org/wiki/Transferrin

Sato, T., Omichi, K., & Ikenaka, T. (1988). Simple assay for sialyltransferase activity with a new fluorogenic substrate. Jounal of Biochemistry, 104, 18-21.

Sialic acid synthesis and signaling. (n.d.). BioFiles, 5(1), 4-7. Retrieved from http://www.sigmaaldrich.com/life-science/learning-center/biofiles/ biofiles-5-1.html

[Sialyllactose]. (2012, December 26). Retrieved from http://www.google.com/patents/EP2536736A1?cl=en

Strynadka, N., & Withers, S. G. (2004, January 26). [Sialyltransferase]. Retrieved from http://pubs.acs.org/cen/topstory/8204/8204notw1.html

ACKNOWLEDGEMENTS

The authors would like to thank the University of New Haven Summer Undergraduate Research Fellowship for financially supporting this research. We would also like to thank Robert Lockwood for his time analyzing the samples using high performance liquid chromatography/triple quadrupole mass spectrometry. Finally, we would like to thank the Forensic Science Department, especially Norma Hollender-Celico, the laboratory manger, for her help making this research happen.