Mechanistic Studies of Tetracycline Monooxygenase

Katelyn Almaraz and Dr. Kevin Francis

Department of Chemistry

TEXAS A&M UNIVERSITY KINGSVILLE®

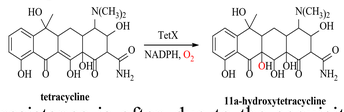
McNAIR SCHOLARS PROGRAM

ABSTRACT

There are three types of resistance mechanisms prokaryotes have evolved to combat the detrimental effects of tetracycline. The most common is efflux, efflux pumps allow microorganisms to regulate their internal environment by removing toxic substances. Another mechanism is ribosomal protection whereby auxiliary proteins are produced to interact with the ribosome and prevent tetracycline binding. The third mechanism, which is not yet fully understood, is enzymatic degradation of tetracycline by recently evolved enzymes produced by bacterium in response to wide-spread use of tetracycline. Tetracycline monooxygenase (TetX) is one of the enzymes involved in this process. It catalyzes the conversion of tetracycline to 11a-hydrotetracycline that is completely unstable at physiological pH and rapidly decomposes before the drug can bind to the ribosome. The long-term goals of the work carried out this summer is to develop a detailed understanding of the chemical mechanism of TetX which is currently unknown. Knowledge of such a mechanism will enable the design of mechanism-based inhibitors of TetX which will abolish its activity and can be used in conjugation with tetracycline to treat bacterial infection. To this end a kinetic assay was developed this summer that will be used in the coming years in the Francis lab. The enzyme was expressed purified, and its steady state kinetic parameters were determined with tetracycline as substrate.

INTRODUCTION

Tetracycline is a very important antibiotic that is not only extremely effective, but readily available and therefore used extensively in agricultural and clinical settings. It binds to the 30S ribosomal subunit preventing amino acyl tRNA from binding to the A site of the ribosome completely preventing translation. While the drug has proved effective in the treatments of bacterial infections its use has declined in modern times due to the emergence of tetracycline resistance that many strains of bacteria have developed in response to the overuse of the drug.



Tetracycline resistance is often due to the acquisition of new genes, which code for energy-dependent efflux of the drug out of the cell or for proteins that protect bacterial ribosomes from the binding of the molecule. A more recently characterized resistance mechanism is the enzymatic degradation of the drug by flavin dependent enzymes. One of the enzymes is tetracycline monooxygenase (TetX). TetX is a flavin adenine dinucleotide (FAD) dependent enzyme that catalyzes the reaction shown above

MATERIALS & METHODS

MATERIALS:

The pET 22a+ plasmid containing the gene encoding for TetX was generously provided by Professor Gerard Wright of McMaster University, (Ontario, Canada). Professor Giovanni Gadda (Georgia State University, Atlanta, GA) provided the DH5α *Escherichia coli* competent cells used to propagate and store the pET22a+-*tet*X2 plasmid. The Clarke-type oxygen electrode used to monitor enzymatic activity was from Hansatech Instruments, Norfolk, United Kingdom. UV Visible absorbance spectra were recorded using a Thermo-Fisher (Waltham, MA) SX 60 scanning spectrophotometer. The resin used for the nickel affinity column was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest purity commercially available.

METHODS:

Streak cells → Overnight Culture→ Subculture, monitor, and induce→ Harvest and lyse cells → Purify with Ni Affinity Column → Analyze by activity and Bradford

The basic protocol that we used, was essentially we grew up the cells, through molecular biology techniques we convince them to make a lot of TetX, we then killed them, broke them open and purified the enzyme.

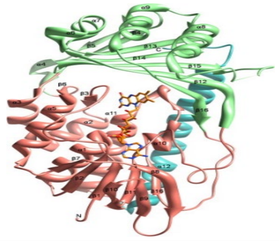


Figure 1: Structure of TetX. Overall Structure of TetX indicating the sub-division into domains by coloring. Elucidation of the chemical mechanism, transition state structure and the roles of specific amino acid residues in catalysis by TetX will guide rational inhibitor designs aimed to combat tetracycline resistance.

RESULTS

Sample	Total Activity ($\mu molO_2/s$)[a]	Total Protein (mg)[b]	Specific Activity (U/mg)[c]	Purification Index
Cell Extract	$(4.3 \pm 0.6) \times 10^4$	1,400	$(30 \pm 5) \times 10^4$	1
40% $(NH_4)_2SO_4$	$(3.8 \pm 0.6) \times 10^4$	70	$(530 \pm 80) \times 10^4$	20
Nickel Affinity	$(1.4 \pm 0.1) \times 10^5$	50	$(2800 \pm 200) \times 10^5$	90

[a]Assay contained 1 mM tetracycline + 50 µM NADPH in 50 mM sodium phosphate at pH 6.0. Assays were conducted in triplicate wand the average value is reported with its standard deviation.
[b]Mass of protein was determined by Bradford assay using bovine serum albumin as a standard protein.
[c]Specific activity was determined by the division of units of activity and total protein.

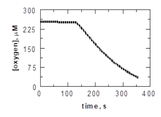


Figure 2: Representative Oxygen Electrode Trace to determine TetX activity. Activity assays of TetX in 50 mM sodium phosphate pH 6.0 and room temperature. The reaction contained 100 µM tetracycline and 50 µM NADPH and was initiated by the addition of the enzyme. Assays were typically repeated in triplicate and the average values are reported in Table 1.

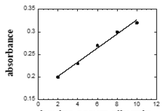


Figure 3 Bradford Assay to Quantify Total Protein. Stock solutions of bovine serum albumin were allowed to incubate with Coomassie Blue for 10 min. The absorbance of the standard solutions was measured at 595 nm and plotted as a function of protein concentration. Fitting the data yielded a line ($y = 0.171 + 0.016x$; $R^2 = 0.989$) that was used to calculate total protein in Table 1.

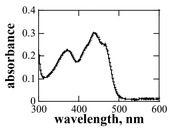


Figure 4 UV-Visible Absorbance Spectrum of Purified TetX activity. The spectrum was recorded in 50 mM sodium phosphate pH 7.0 using a Thermo Fisher SX 60 scanning spectrophotometer. The peak at 444 nm was used to calculate the concentrations of TetX 24.72 µM.

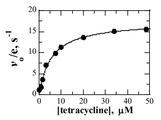


Figure 5: Steady State Kinetics of TetX with Tetracycline as substrate. Assays were conducted with 100 µM NADPH and atmospheric O_2 at pH 6.0 and room temperature. Data were fit to eq 1 to determine the kinetic paramours of the enzyme ($R^2 = 0.992$).

Clean and reproducible traces were observed after significant effort was put into determine the appropriate order of substrate and cofactor addition. This allowed for lower errors in the purification table shown above and more importantly, to achieve the goal of the summer project, which was to determine the steady state kinetic parameters of the enzyme by constructing a Michaelis-Menten curve

The Bradford assay constructed to quantify the mass of protein at each stage of the purification is shown in Figure 3. As expected, using a the fully established protocol, yielded larger amounts of TetX than even what was reported in the published report from the Francis group.[6] The data in Table 1 revealed that the enzyme was purified 90-fold. Most importantly 50 mg of highly pure, very active TetX was obtained, aliquoted and is ready for use by other students in the 2021-2022 academic year.

Since the goal of this project was to measure the steady state kinetics of TetX, its UV-visible absorbance spectrum was measured to calculate a more accurate concertation using the Lambert-Beer law using the molar extinction coefficient of FAD (Figure 4). The spectrum of TetX had peaks centered at 444 and 364 nm typical for the isoalloxazine ring of the FAD cofactor. A concentration of 24.72 µM. This experiment represents the first measurement of the UV-visible absorbance spectrum in the Francis lab, and it agrees well with the results reported by Yan *et al*.

After the enzyme was purified, the activity assay was optimized and the concentration of the TetX solution was determined with reasonable accuracy, the steady state kinetic parameters of the enzyme were determined for the very first time at Texas A &M University-Kingsville. The experiment was carried out at pH 6.0 and the results are shown in Figure 5. A k_{cat} value of 17.4 ± 0.6 s^{-1} was determined as well as specificity constant of 280,000 ± 20, 000 $M^{-1}s^{-1}$. While a single kinetic curve does not reveal significant insights into the mechanism of TetX, these results and the optimization of the activity assay for TetX provide a milestone in the project carried out in the Francis lab.

REFERENCES

1. Chopra, I.; Roberts, M., Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **2001,** *65* (2), 232-60 ; second page, table of contents.

2. Connell, S. R.; Tracz, D. M.; Nierhaus, K. H.; Taylor, D. E., Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob Agents Chemother* **2003,** *47* (12), 3675-81.

3. Yang, W.; Moore, I. F.; Koteva, K. P.; Bareich, D. C.; Hughes, D. W.; Wright, G. D., TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem* **2004,** *279* (50), 52346-52.

4. Forsberg, K. J.; Patel, S.; Wencewicz, T. A.; Dantas, G., The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes. *Chem Biol* **2015,** *22* (7), 888-97.

5. Bernal, J., Ojutiku, Zainabb, Cordero, Adriana, Martinez, AshLee, Berkey and Francis, Kevin, An Improved Protocol for the Expression and Purification of Tetracycline Monooxygenase: An Enzyme Involved in Antibiotic Resistance. *Annals of Advanced Biomedical Science* **2020,** *3*, 2.

6. Inoue, H.; Nojima, H.; Okayama, H., High efficiency transformation of Escherichia coli with plasmids. *Gene* **1990,** *96* (1), 23-8.

7. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **1976,** *72*, 248-54.

8. Macheroux, P.; Massey, V., 8-thiocyanatoflavins as active-site probes for flavoproteins. *Biochemistry* **1991,** *30* (2), 456-64.

ACKNOWLEDGEMENTS

Former Students

•Zainab Ojutitku (M.S.)
•Jennifer Bernal
•Adriana Cordero
•AshLee Martinez
•Kaylee Berkey
•Jason Bakonyi
•Niles MacFarland
•Kayla Leftwhich

Special thanks to

•Alejandra Amaya
•James Remelius
•Dr. Timothy Oblad
•Dr. Kevin Francis

Funding

Robert A Welch Foundation AC-00006
Ronald E. McNair program
University Research Award TAMUK