Structural Analysis of Aromatic Polyketide Synthase for the Design of Novel Aromatic Polyketide Natural Products of Pharmaceutical Importance

I. PURPOSE, OBJECTIVE AND APPROACH

Nature creates a huge array of natural products that are diverse in their chemical structures and bioactivity. One such example is the polyketides, a large family of natural products that are an extremely rich source of bioactive molecules (1,2). Representative compounds include cholesterol lowering drugs (such as lovastatin) (3), antibiotics (such as tetracyclines and actinorhodin) and anticancer agents (such as doxorubicin) (Fig.1A) (1,2). The biosynthesis of polyketides is catalyzed by a multifunctional enzyme known as polyketide synthase (PKS). Although the chain elongation steps catalyzed by PKS resemble the action of fatty acid synthase (FAS), polyketides are subjected to different chain modifications (5). As a result, a huge diversity of polyketides can be produced biosynthetically. Combination of polyketide subunits can potentially lead to billions of polyketide analogs as novel drug leads. However, this has been severely hampered by the lack of molecular information about PKS subunits. Therefore, studying the structure-function relationship of key enzymes is essential for the design of new polyketide compounds as novel drug leads (4).

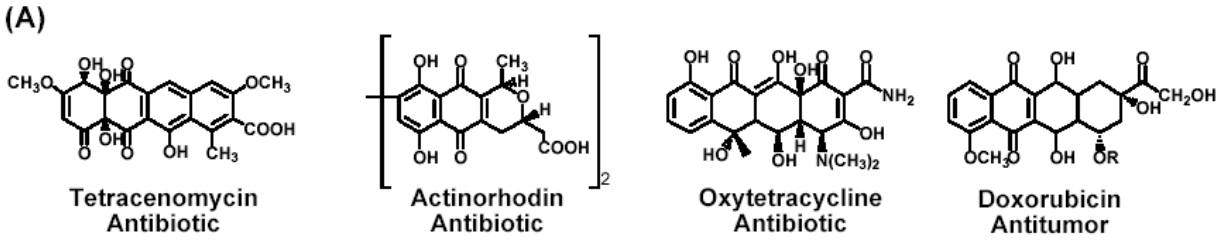

Fig. 1: (A) Polyketide Natural Products of pharmaceutical importance.

A. Purpose

There are 3 types of different PKSs (8). Our purpose in the Tsai lab is to study Type II or "aromatic" PKSs, which synthesize aromatic polyketides such as actinorhodin and tetracycline (Fig. 1A). Aromatic PKSs are comprised of 5-10 distinct enzymes whose active sites are used iteratively in the chain elongation cycle (9,10). The polyketide chain is covalently linked to acyl carrier protein (ACP). Following iterative chain elongation by the ketosynthase (KS) / chain length factor (CLF) heterodimer, the first ring is formed either uncatalyzed, in the active site of KS/CLF or in the active site of KR, leading to the intermediate **1** (Fig. 1B). The polyketide chain is then reduced at the C9 position by ketoreductase (KR) to form intermediate **2**, followed by subsequent aromatic ring formations catalyzed by aromatase and cyclase (11). Although reduction of the carbonyl group in polyketides is similar to the reduction in fatty acid chains, the polyketide reductase (KR) has a high specificity for the C9-carbonyl group (12,13). Subsequently the cyclization reaction catalyzed by aromatase (ARO) downstream of KR is also regio-specific and related closely to the C9 reduction (11). As part of the PKS team, my purpose is to understand the molecular basis of KR and ARO regio- and stereo-specificity (4) by solving the structure of these enzymes and studying the protein-protein and protein-substrate interaction. So far, we have successfully crystallized and solved the crystal structures of cofactor-bound KR and apo ARO, resulting in the publication of our result in Biochemistry and the preparation of two more manuscripts. In all three articles, I am the major contributor of the work**.** Based on these results, the following objectives are proposed.

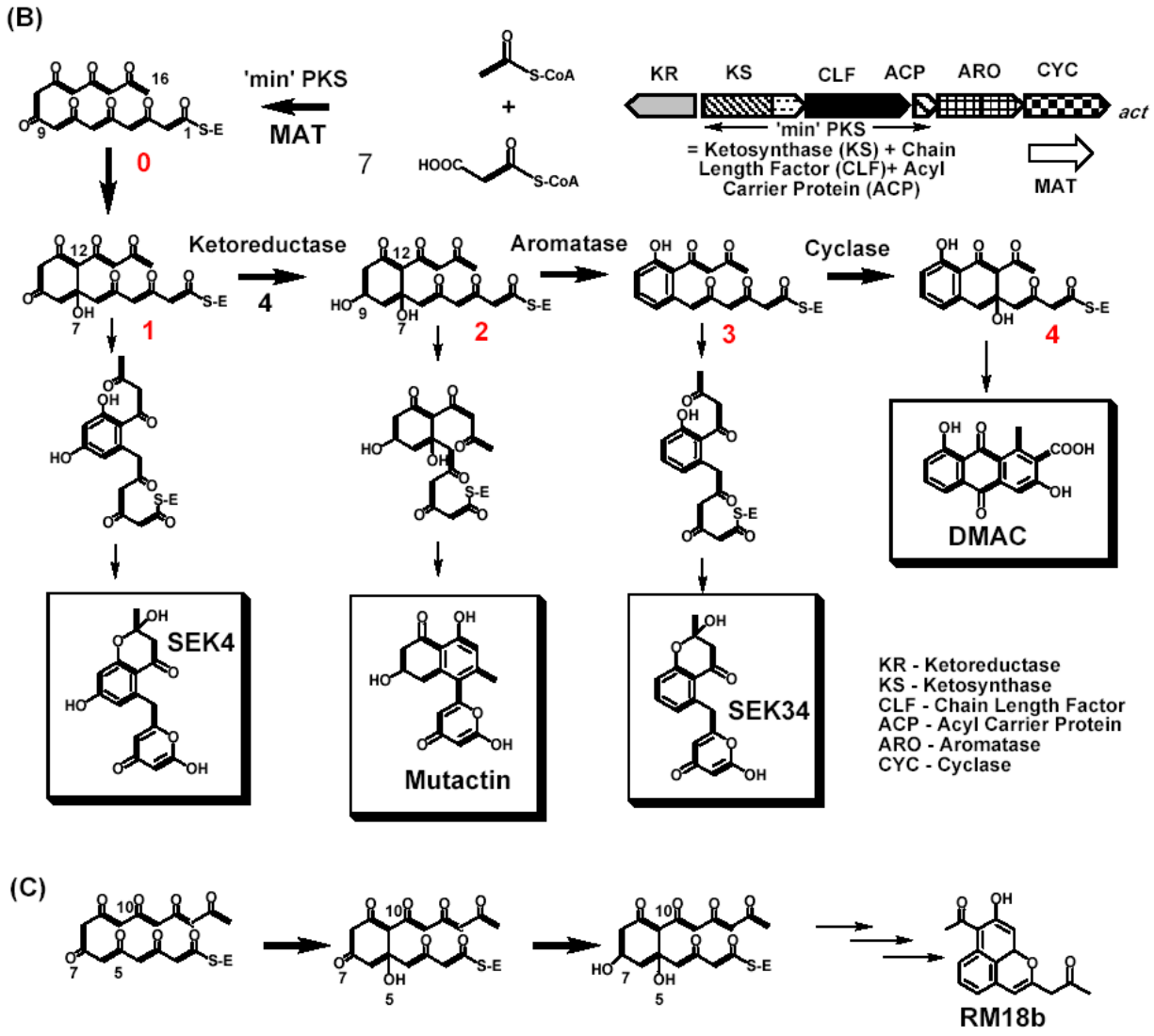

Fig. 1(B) The biosynthesis of actinorhodin. (C) Alternative cyclization between C5-C10 leads to the reduction at C7 position and the eventual product RM18b.

B. Objectives

The main objective of this research project is to solve the crystal structures of wild type and mutant's actinorhodin ketoreductase (actKR) and tetracenomycin aromatize (tamari) that is bound with substrates or inhibitors. With the co-crystal structures, in comparison with the ape structures that we had obtained, we will be able to study the nature of substrate binding in the active sites, which accounts for the region- and sterol-specificity of these two enzymes. Understanding the substrate binding of these enzymes bridges the gap in current PKS research, and it is also of great importance for combinatorial polypeptide biosynthesis (4). As presented in our recent paper in Biochemistry, we find actor tetramer containing a Crossman fold (14), which consists of repeated alpha-beta motifs (Fig.2A). Substrate-docking simulation of the polypeptide substrate into the substrate-binding pocket revealed that among the eight carbonyl groups that are possible for ketoreduction (Fig. 1B), the C9-carbonyl is in the optimum position for enzyme-mediated reduction without evoking extensive conformational change. Simulations to dock other carbonyl groups at positions other than the C9-carbonyl shows extensive steric clashes (4). Achieving our first objective on solving the substrate/inhibitor co-crystal structures of actKR will enable us to dissect the stereo- and regiospecificity of this important polyketide modifying enzyme.

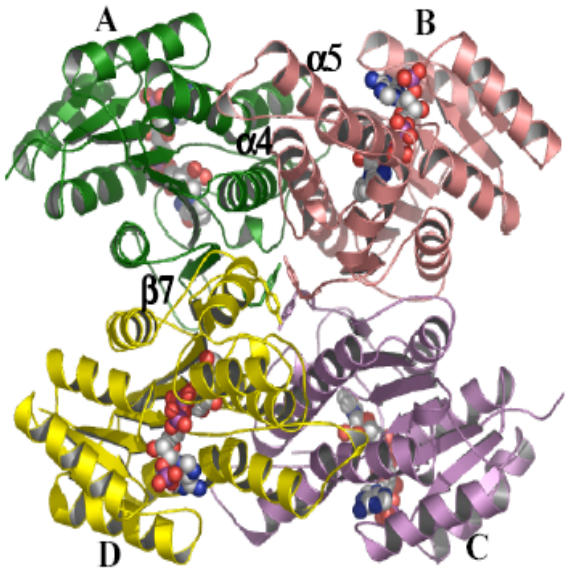

Fig.2A Structure of actKR

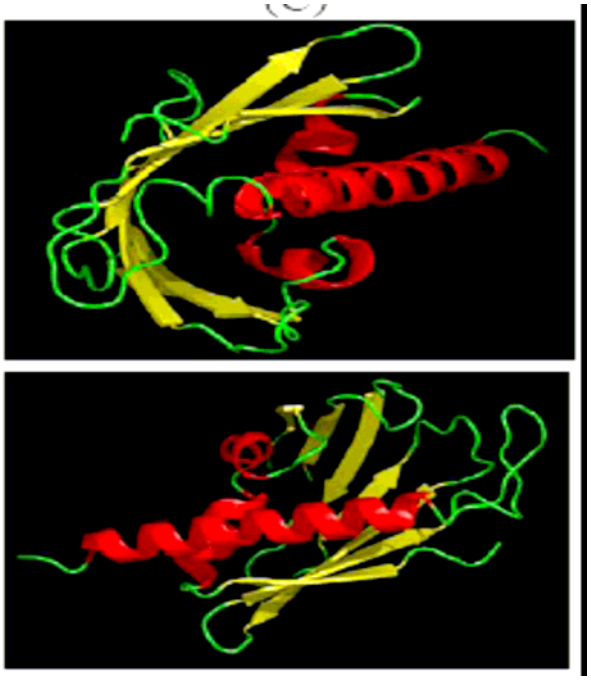

Figure 2B Ribbon structure of tcmARO portraying a “hot dog in a bun”

Similarly, the structure of tcmARO has been diffracted to a resolution of 1.7 angstroms. Refinement of x-ray diffraction data has resulted in the secondary structure of tcmARO shown above (Fig.2B). Docking simulations with a small molecule ligand shows that the major docking site is at the dimeric interface of the protein. Studies of the residues within the binding pocket shows that most are hydrophobic residues. Furthermore, the electropositive surface of the binding pocket facilitates the interaction between the protein and carbonyl groups of the substrate. By identifying the residues in the pocket, we can advance our studies to do site-directed mutagenesis on the residues to see how the mutations affect substrate binding as well as product outcome. .

C. Approach

i.Protein Production of tcmARO and actKR

Expression, harvesting, and purification of tcmARO and actKR will be necessary for the downstream experiments; to crystallize and perform assays on the mentioned protein.

ii. Crystallization

Proteins will be crystallized under certain solvent conditions with co-factors, substrates and inhibitors. Crystals of good quality will be taken to diffract.

iii. Data Analysis

The results of the crystal diffraction of the enzymes will be further analyzed and refined using the software DENZO and CNS, which will provide an electron density map of the structure. Protein model can then be fitted into the electron density map to form a complete structure. The active site and how the cofactor and substrate bind within the enzyme will be determined. Further work can be done to mutate the active site for possibility of novel versions of the enzyme and its ability to bind substrates.

iv. Site-directed mutagenesis

Certain residues of the active site in tcmARO and actKR will be mutated using commercially available mutagenesis kits. Proposed mutations and expected products will be previously viewed with docking programs.

v. in vivo studies of polyketide biosynthesis

Mutated genes of the two proteins will be inserted into a plasmid along with the genes for the complete minimal PKS to work. The plasmid will be inserted into a vector that can support polyketide biosynthesis. Transformation and expression will be performed and cultures will be fed with incoming substrate. The final polyketide product will be harvested, separated, and analyzed with NMR.

II. Responsibilities and Timeline

A. Responsibility

Under the direction and guidance of Dr. Tsai, as well as Tyler Korman and Brian Ames who are graduate students in the PKS team, I will be responsible for the continued protein production of actKR and tcmARO. Although crystal structures have been solved for both enzymes, there is a continued necessity for pure protein to be available for further crystallization with substrates, inhibitors, and for use in assays. My responsibility from now (Winter Quarter 2005) until summer is to search for a substrate that can be co-crystallized with tcmARO. With the necessary funding provided by ID-SURE and SURP grants, these various substrates can be purchased and co-crystallized with wild type tcmARO. Ideal protein crystals will then be separated, coated in a cryoprotectant and freeze with liquid nitrogen to be diffracted. With the essential crystals, I hope to accompany Dr. Tsai and the graduate students in the team for a trip to Berkeley's Advanced Light Source synchrotron to collect data for my crystals. Further training will be given by Dr. Tsai on x-ray crystal diffraction and the use of the various computer programs for data analysis.

My other responsibility is to learn how to perform site-directed mutagenesis on the residues within the binding pocket of tcmARO. Mutations of these residues have been proven to not affect the overall folding of the enzyme and the protein-protein interaction with other subunits of PKS.

B. Timeline for Summer 2005

- June 2005
  * Protein expression, harvesting and purification of wild-type tcmARO to a certain degree of purity for successful crystallization.
  * Crystallize the apo-wt tcmARO under the crystallization which produced crystals before to see if crystals are reproducible under the given conditions.
  * Purchase of substrates for co-crystallization.

- July 2005
  * Crystallization of tcmARO with specified substrates and monitoring of conditions in which crystallization is optimum
  * Ideal crystals will be taken to diffract and data will be collected, analyzed and refined by Dr. Tsai and graduate students. I will be learning the background and basics of x-ray crystallography.

- August 2005
  *Ongoing training will be given by Dr. Tsai on how to perform mutagenesis protocols on the residues in the binding pocket of tcmARO.
  *Finalize results in preparation for upcoming May Symposium.

III. References

1. Hopwood,D.A. (1997) Genetic Contributions to Understanding Polyketide Synthases, *Chem Rev 97*, 2465-2498.
2. Staunton, J., and Weissman, K.J. (2001) Polyketid biosynthesis: a millennium review, *Nat Prod Rep 18*, 380-416.
3. Tobert, J.A. (2003) Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors, *Nat Rev Drug Discov* 2, 517-526.
4. Korman, T.P., Hill, J.A., Vu, T.N., and Tsai, S.C. (2004) Structural Analysis of Actinorhodin Polyketide Ketoreductase: Cofactor Binding and Substrate Specificity, *Biochemistry (under publication).*
5. O'Hagan, D. (1993) Biosynthesis of fatty acid and polyketide metabolites*, Nat Prod Rep 10,* 593-624.
6. Walsh, C.T. (2004) Polyketide and nonribosomal peptide antibiotics: modularity and versatility*, Science 303*, 1805-1810.

7. Nicholson, T.P., Winfield, C., Westcott, J., Crosby, J., Simpson, T.J., and Cox, R.J. (2003) First in vitro directed biosynthesis of new compounds by a minimal type II polyketide synthase: evidence for the mechanism of chain length determination, *Chem Commun (Camb),* 686-687.
8. Shen, B. (2003) Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms, *Curr Opin Chem Biol 7*, 285-295.
9. Axford, L. C., Simpson, T. J., and Willis, C. L. (2004) Synthesis and incorporation of the first polyketide synthase free intermediate in monocerin biosynthesis, *Angew Chem Int Ed Engl 43*, 727-730.
10. McDaniel, R., Ebert-Khosla, S., Hopwood, D. A., and Khosla, C. (1995) Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits, *Nature 375*, 549-554.
11. McDaniel, R., Ebert-Khosla, S., Hopwood, D. A., and Khosla, C. (1995) Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits, *Nature 375*, 549-554.
12. McDaniel, R., Ebert-Khosla, S., Fu, H., Hopwood, D. A., and Khosla, C. (1994) Engineered biosynthesis of novel polyketides: influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase, *Proc Natl Acad Sci U S A 91*, 11542-11546.
13. Hertweck, C., Xiang, L., Kalaitzis, J. A., Cheng, Q., Palzer, M., and Moore, B. S. (2004) Contextdependent behavior of the enterocin iterative polyketide synthase; a new model for ketoreduction, *Chem Biol 11*, 461-468.
14. Rossmann, M. G., and Argos, P. (1981) Protein folding, *Annu Rev Biochem 50*, 497-532.
15. Tsai, S.C. (2005) CRCC Research Proposal
16. Ames, B. (2004) Structual Analysis of tcmARO/CYC. *Fall 2004 Rotational Report*- UCI Department of Molecular Biology, Biochemistry, and Genetics.