Normalized activities were analyzed by a clustering statistical algorithm [9] that segregated the enzymes into four groups based on similar substrate specificities. In an interesting twist that sought relationships among substrates in chemical space, correlations between substrates were used to identify groups of substrates with similar chimera profiles. This approach addresses whether a chimera's activity on one substrate predicts activity on another with the outcome being the identification of substrates that can be surrogates for other substrates in the same group. This classification has the potential to facilitate discovery of useful catalysts by high-throughput screening using these surrogates. Not too surprisingly, a correspondence was found between the protein clusters and the substrate clusters. The analysis of the similarities and differences in physical, structural, and chemical properties within and between clusters—either protein or substrate—should be a powerful tool for gaining insight into P450 function and for predicting the enzymatic activity of untested chimeras on tested substrates and tested chimeras on untested substrates. The authors use their analysis to suggest that swapping of the reductase domains always resulted in functional enzymes because key interdomain interactions were conserved.

The results illustrate that diversity in sequence leads to diversity in function. The less expected, but more important finding is that such diversity in function need not come at the expense of absolute activity. For all eleven substrates, the top-performing chimeric enzyme's activity exceeded that of the three parental enzymes. However, the work most importantly speaks to the power of directed evolution approaches as a tool for not just exploring, but for analyzing sequence space and its relationship with functional space. Statistical analysis of such combinatorial protein engineering experiments, among other approaches, will allow us to move beyond simply cataloging protein space, but understanding it as well.

REFERENCES

1. Landwehr, M., Carbone, M., Otey, C.R., Li, Y., and Arnold, F.H. (2007). Chem. Biol. *14*, 1–2.
2. Borges, J.L. (1962). Labyrinths: Selected Stories and Other Writings, D.A. Yates and J.E. Irby, eds. (New York: New Directions), pp. 51–58.
3. Arnold, F.H. (2000). Adv. Protein Chem. *55*, ix–xi.
4. Keefe, A.D., and Szostak, J.W. (2001). Nature *410*, 715–718.
5. Bloom, J.D., Labthavikul, S.T., Otey, C.R., and Arnold, F.H. (2006). Proc. Natl. Acad. Sci. USA *103*, 5869–5874.
6. Otey, C.R., Landwehr, M., Endelman, J.B., Hiraga, K., Bloom, J.D., and Arnold, F.H. (2006). PLoS Biol. *4*, e112.
7. Ostermeier, M. (2003). Trends Biotechnol. *21*, 244–247.
8. Voigt, C.A., Martinez, C., Wang, Z.G., Mayo, S.L., and Arnold, F.H. (2002). Nat. Struct. Biol. *9*, 553–558.
9. Larsson, A.K., Emren, L.O., Bardsley, W.G., and Mannervik, B. (2004). Protein Eng. Des. Sel. *17*, 49–55.

Antifungal Tradecraft by Cholesterol Oxidase

Natasha M. Nesbitt[1] **and Nicole S. Sampson**[1,*]
[1] Department of Chemistry, Stony Brook University, Stony Brook, NY 11794, USA
*Correspondence: nicole.sampson@stonybrook.edu
DOI 10.1016/j.chembiol.2007.03.003

In this issue of *Chemistry & Biology*, Aparicio and coworkers report that secreted bacterial cholesterol oxidase is required for the biosynthesis of the antifungal polyene pimaricin by *Streptomyces natalensis* [1]. Their discovery expands the inventory of tasks this biotechnologically important enzyme performs.

Bacterial cholesterol oxidases have been utilized by humans for more than 30 years to analyze the bane of Western eating habits, serum cholesterol [2–4]. For their own use, bacteria, actinomycetes in particular, secrete cholesterol oxidase to metabolize 3β-hydroxy sterols to 3-ketosteroids, which serve as their energy source [5, 6]. Cholesterol oxidase, more properly 3β-hydroxysterol oxidase, is the first enzyme in that metabolic pathway that yields propionate and acetate as the ultimate products. Since their first isolation, cholesterol oxidases have been rediscovered in screens for insecticides [7], and have been widely used in the search for lipid rafts [8]. In the presence of a sufficient quantity of either cholesterol or ergosterol mixed with saturated lipids, liquid-phase membranes are ordered, that is, they can form lipid rafts. Oxidation of the sterol to cholest-4-en-3-one or ergosta-4,7,22-trien-3-one (Figure 1) results in dissolution of these lipid domains. These liquid-disordered membranes render the lipid bilayer more permeable and susceptible to lysis, which is the mechanism of insecticidal activity [9].

Now, Aparicio and coworkers report a new function for the *Streptomyces*

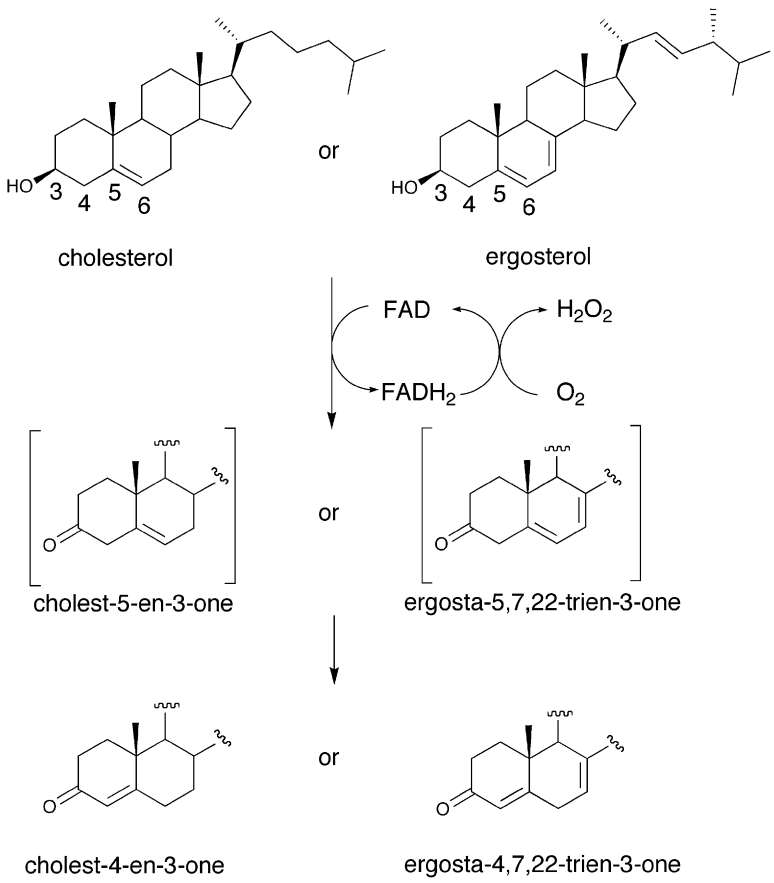

Figure 1. The Reaction Catalyzed by 3β-Hydroxysterol Oxidases
In the reaction catalyzed by cholesterol oxidase, the 3β-hydroxysterol (cholesterol or ergosterol) is oxidized and isomerized to an α,β-unsaturated ketone.

natalensis cholesterol oxidase PimE [1]. The *pimE* gene resides in the center of the pimaricin polyketide biosynthetic cluster in the *S. natalensis* genome. It has been a mystery whether its residence in the gene cluster was an accident of evolution or a consequence of a functional role in pimaricin biosynthesis, since the synthesis of the polyketide does not require cholesterol oxidation. The authors discovered that disruption of the *pimE* gene inhibits pimaricin production, suggesting that the gene has a functional role in antifungal biosynthesis.

Cholesterol oxidases catalyze the FAD-dependent oxidation of a 3β-hydroxy sterol to a 3-keto steroid and hydrogen peroxide. The β,γ-unsaturated ketone intermediate undergoes a 1,3 allylic isomerization to form an α,β-unsaturated ketone (Figure 1). Detailed kinetic analysis and site-directed mutagenesis have identified 3 active site residues, His, Glu, and Asn, as important for oxidation and isomerization [10, 11]. The proposed functions of these amino acids in catalysis is supported by crystallographic analysis [12–14] and homology with other members of the *g*lucose-*m*ethanol-choline (GMC) oxidoreductase family [15]. The primary amino acid sequence of PimE has a high percent identity (59%–91%) with known cholesterol oxidases. Among the conserved amino acids are the essential His, Glu, and Asn, as well as the consensus sequence for binding of the flavin cofactor.

Aparicio and coworkers isolated and purified PimE from *S. natalensis* culture filtrates and demonstrated that it is truly an extracellular cholesterol oxidase [1]. The enzyme produces hydrogen peroxide and cholestenone with a catalytic efficiency consistent with it being a cholesterol oxidase, and it has a molecular mass consistent with that predicted for the mature PimE according to SDS-PAGE analysis. Although no spectral data were presented, it is presumed that a flavin cofactor is bound to the protein, as the sequence homology for the flavin-binding domain is high. The authors disrupted the *pimE* gene and found that culture broths of the mutant (*S. natalensis* D3) were unable to catalyze cholesterol oxidation. Unexpectedly, pimaricin production was blocked in the mutant strain. No pimaricin could be isolated from culture filtrates nor was antifungal activity against *Candida utilis* observed (Figure 4 in [1]).

Cholesterol oxidase activity and pimaricin production were restored in the *S. natalensis* D3 mutant complemented with a vector containing *pimE* and its putative promoter. More surprisingly, adding purified PimE protein to the D3 mutant cultures restored pimaricin biosynthesis. Moreover, other commercially available cholesterol oxidases restored pimaricin production in the D3 mutant. Interestingly, the cholesterol oxidase from *Pseudomonas* (now *Burkholderia cepacia* [16]), that is not a member of the GMC oxidoreductase family and does not share sequence or structural homology, complemented the mutant very poorly. The weak complementation suggests that the protein's structure may be important for pimaricin induction.

The precise mechanism by which extracellular PimE induces pimaricin biosynthesis remains to be elucidated. Transcriptional regulation of the pimaricin cluster was ruled out as transcription of the cluster genes was not affected by *pimE* deletion [1]. Thus, it appears that PimE controls either translation of the pim gene cluster or secretion of pimaricin. Two mechanistic models for upregulation of pimarcin production seem plausible. First, the product generated by PimE may be the pimaricin inducer, or second, PimE itself may activate pimaricin production by acting as a ligand for a receptor signaling system.

The authors further investigated the importance of sterol oxidation for polyketide production. Addition of the

products from an oxidase reaction (H_2O_2 and cholest-4-en-3-one) to the D3 mutant did not induce pimaricin production. However, preincubation of PimE with cholesterol or ergosterol before addition to D3 cultures suppressed PimE's induction of pimaricin biosynthesis. Furthermore, inhibition of PimE catalytic activity with $HgCl_2$ or $AgNO_3$ before addition to D3 cultures also prevented pimaricin production. The exact mechanism of PimE inactivation is unknown—perhaps oxidation of a cysteine or methionine blocks substrate binding [17].

The authors' experiments suggest that sterol oxidation products are not directly involved in pimaricin production, yet oxidase activity is required for induction. There are caveats to these experiments, and additional work is required to fully understand the regulatory mechanism. For example, the relative substrate specificities of PimE for different sterols have not been assessed under physiological conditions, and the identity of the true substrate is not yet known. The physiological PimE product may be ergosta-4,7,22,trien-3-one or an as yet unidentified ketone product. Consequently, addition of the correct product could potentially induce pimaricin production. Moreover, addition to bacteria of product solubilized in a detergent micelle, as Aparicio and coworkers did [1], may have a very different effect than direct formation of the product in the bacterial membrane. Interestingly, D3 cultures grown in mineral medium lacking possible sterols still produced pimaricin when complemented with PimE, albeit at very low levels compared to complementation of D3 cultures grown in yeast/malt extract. PimE induction of pimaricin biosynthesis in yeast-free media argues against the sterol product hypothesis. However, there is a possibility that a sufficient quantity of sterol copurified with the enzyme when it was isolated from *S. natalensis* cultures to account for the low level of pimaricin production.

The alternative to pimaricin induction by the PimE reaction product is a receptor-mediated signaling mechanism. In this mechanism, binding of the PimE protein to a receptor in the *Streptomyces* cell wall would initiate a signal to produce pimaricin. As no direct evidence for a PimE receptor has yet been obtained, the type of signaling molecules that might be involved are unknown. Additionally, signaling by PimE could require that a ligand be bound to the enzyme, e.g., a sterol or another unknown compound. The low complementation activity by the structurally distinct *Pseudomonas* (*Burkholderia*) cholesterol oxidase supports the receptor hypothesis.

A mechanism featuring PimE as a sterol sensor is appealing, and sensing could be operating in either model. *Streptomyces* do not typically have sterols in their membranes. However, the presence of a fungal competitor, e.g., *Candida*, may result in transfer of ergosterol or some other sterol into the *Streptomyces* membrane. Perhaps the presence of ergosterol is a signal to *Streptomyces* to initiate PimE expression, and thus, polyketide production. Even more speculatively, the expression of PimE could serve the dual purpose of membrane structure modulation and stimulation of pimaricin production. If fungal sterols were incorporated into the *Streptomyces* membrane, they would rigidify it and interfere with secretion and/or signaling. Oxidation of sterol would help restore the correct fluidity to the membrane. Linking pimaricin production to sterol sensing is attractive because the polyene macrolide interacts with ergosterol in the fungal membrane [18]. Thus, pimaricin biosynthesis would only occur if the direct target, ergosterol, were present.

Identification of the environmental signal(s) that induces PimE expression is key to understanding PimE function. The expression of all previously identified cholesterol oxidases is induced by the presence of 3β-hydroxysterol in the culture medium. If PimE expression is sterol-induced, this finding will provide strong support for an environmental sensing role for PimE. PimR has been identified as a positive regulator of pimaricin biosynthesis and *pimE* transcription [19]. However, what regulates PimR and its relationship to *pimE* are unclear.

Aparicio and colleagues present intriguing data that an extracellular enzyme induces polyketide biosynthesis. Putative cholesterol oxidases have been identified within the biosynthetic gene clusters of the antifungal polyketides filipin (*pteG*) and CE-108/rimocidin (*rimD*) [20, 21] produced by other *Streptomyces* strains. All of these polyketides are elicited against fungi, whose membranes are distinctly different than bacterial membranes by virtue of their ergosterol content, making the pimaricin regulatory model an attractive paradigm that may be more widely utilized. It will be exciting to watch the cholesterol oxidase story continue to unfold and to learn the molecular details of antifungal biosynthesis regulation.

REFERENCES

1. Mendes, M.V., Recio, E., Antón, N., Guerra, S.M., Santos-Aberturas, J., Martín, J.F., and Aparicio, J.F. (2007). Chem. Biol. *14*, this issue, 279–290.
2. Richmond, W. (1973). Clin. Chem. *19*, 1350–1356.
3. Fukuda, H., Kawakami, Y., and Nakamura, S. (1973). Chem. Pharm. Bull. (Tokyo) *21*, 2057–2060.
4. Smith, A.G., and Brooks, C.J.W. (1974). J. Chromatogr. *101*, 373–378.
5. Tak, J. (1942). Antonie Van Leeuwenhoek *8*, 32–40.
6. Turfitt, G.E. (1944). Biochem. J. *38*, 492–496.
7. Corbin, D.R., Grebenok, R.J., Ohnmeiss, T.E., Greenplate, J.T., and Purcell, J.P. (2001). Plant Physiol. *126*, 1116–1128.
8. Lange, Y., Ye, J., and Steck, T.L. (2007). Biochemistry *46*, 2233–2238.
9. Ghoshroy, K.B., Zhu, W., and Sampson, N.S. (1997). Biochemistry *36*, 6133–6140.
10. Sampson, N.S., and Kass, I.J. (1997). J. Am. Chem. Soc. *119*, 855–862.
11. Kass, I.J., and Sampson, N.S. (1998). Biochemistry *37*, 17990–18000.
12. Yin, Y., Sampson, N., Vrielink, A., and Lario, P. (2003). J. Mol. Biol. *326*, 1635–1650.
13. Ye, Y., Lario, P., Vrielink, A., and Sampson, N.S. (2001). Biochemistry *40*, 13779–13787.
14. Yue, K., Kass, I.J., Sampson, N., and Vrielink, A. (1999). Biochemistry *38*, 4277–4286.
15. Cavener, D.R. (1992). J. Mol. Biol. *223*, 811–814.
16. Doukyu, N., and Aono, R. (2001). Appl. Microbiol. Biotechnol. *57*, 146–152.

17. Loubat-Hugel, C., Tritsch, D., and Biellmann, J.F. (1994). C. R. Acad. Sci. III *317*, 299–303.

18. Gale, E.F. (1984). Macrolide Antibiotics, S. Omura, ed. (Orlando, FL: Academic Press), pp. 425–455.

19. Anton, N., Mendes, M.V., Martin, J.F., and Aparicio, J.F. (2004). J. Bacteriol. *186*, 2567–2575.

20. Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., et al. (2001). Proc. Natl. Acad. Sci. USA *98*, 12215–12220.

21. Seco, E.M., Perez-Zuniga, F.J., Rolon, M.S., and Malpartida, F. (2004). Chem. Biol. *11*, 357–366.

When an Inhibitor Promotes Activity

Michel Bouvier[1,*]
[1] Université de Montréal, Institute for Research in Immunology and Cancer, Marcelle-Coutu Pavillion, C.P. 6128 Succursale Centre-Ville, Montreal, Quebec H3C 3J7, Canada
*Correspondence: michel.bouvier@umontreal.ca
DOI 10.1016/j.chembiol.2007.03.004

In a recent issue of *Chemistry & Biology*, Tropak et al. [1] reported a high-throughput screening assay to identify β-hexosaminidase inhibitors that act as pharmacological chaperones and are potential drug candidates for the treatment of Tay-Sachs disease.

Genetic conformational diseases are often caused by modest mutations in proteins that lead to unnecessary recognition of the mutant protein by the endoplasmic reticulum (ER) quality control system as misfolded. These mutations classically occur outside of the predicted functional domain of the affected proteins such that interventions to allow their release from the quality control system and their targeting to their proper site of action could restore function [2]. Classic examples of such diseases include cystic fibrosis, nephrogenic diabetes insipidus, hypogonadotropic hypogonadism, and various lysosomal storage disorders such as GM2 gangliosidosis, Fabry, Gaucher, and Morquio B diseases.

Pharmacological chaperones are small molecules that can bind and stabilize the folding intermediates of the mutant proteins thus favoring their proper folding, ER export, and subsequent trafficking to their normal subcellular location [3]. In most instances, pharmacological chaperones that can rescue folding, trafficking, and function of mutant proteins have been found among ligands that were already known to bind to the affected proteins thus conferring a measure of selectivity. For example, specific receptor antagonists in the case of diseases resulting from mutations in G protein-coupled receptors (like V2-vasopressin and gonadotropin-releasing hormone receptors for nephrogenic diabetes insipidus and hypogonadotropic hypogonadism, respectively [4,5]) and competitive enzyme inhibitors for lysosomal storage disorders [6] were found to increase the levels of active receptors and enzymes in cellular systems, indicating that such compounds could have therapeutic value. Consistent with this notion, a small scale clinical trial carried out in nephrogenic diabetes insipidus patients confirmed the therapeutic activity of a pharmacological chaperone, as administration of a vasopressin antagonist significantly improved kidney functions in five patients harboring three distinct mutations [7].

The use of an antagonist or inhibitor to treat a disease resulting from a loss of function mutation may appear contradictory, but is based on the idea that the high affinity binding of these compounds to the mutant protein provides sufficient interaction time to promote proper folding and escape from the ER quality control system. Obviously, the inhibitors then need to dissociate from the rescued enzyme/receptor to permit their activity. It follows that detailed pharmacokinetic studies are needed to find a drug administration regimen that would result in the optimum therapeutic activity of individual pharmacological chaperones.

Based on the idea that inhibitors can act as pharmacological chaperones and rescue the activity of protein function, Tropak et al. [1] developed a real time, high-throughput assay to identify new inhibitors of human β-hexosaminidase that could have pharmacological activity in the treatment of two forms of GM2 gangliosidosis, infantile Tay-Sachs disease (ISD) and adult Tay-Sachs disease (ATSD). These diseases result from mutations in the gene encoding β-hexosaminidase that render the enzyme unstable most likely due to misfolding, thus making them potential targets for the therapeutic action of pharmacological chaperones. The fluorescence-based in vitro assay developed by the authors uses purified enzyme and identified 24 confirmed inhibitors of the enzymatic activity out of a commercially available 50,000 compound library. Three of these compounds, belonging to distinct classes of chemical structures, were then selected to study their potential action as pharmacological chaperones in treatment of Tay-Sachs disease.