Pfeifer Lab Journal Club

DOI: 10.1002/cbic.200500261

Direct Conversion of Ethane to Ethanol by Engineered Cytochrome P450 BM3

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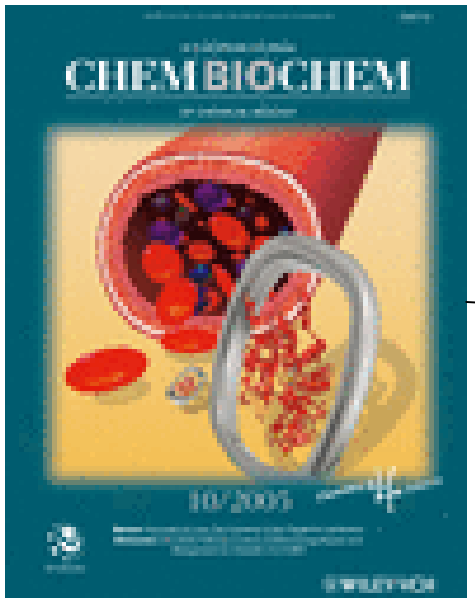
April 5, 2006

Outline

- Background
  - Journal & Corresponding Author
  - Biological Engineering
  - Molecular Evolution & Computational Biophysics
- Motivation
- Previous Work
- Experimental Methods
- Results
- Conclusions & Discussion

ChemBioChem

- In 2004, *ChemBioChem* had an ISI index of 3.5
- A John Wiley & Sons, Inc. journal



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Direct Conversion of Ethane to Ethanol by Engineered Cytochrome P450 BM3

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http://www3.interscience.wiley.com/cgi-bin/jissue/112098159

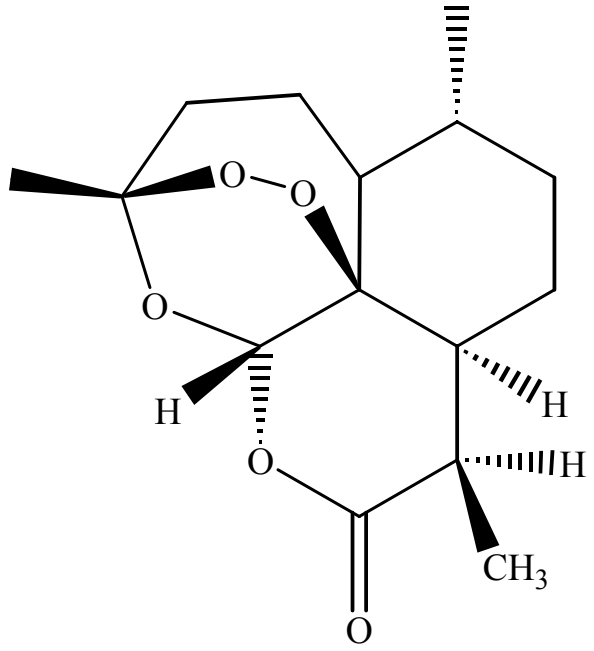
Frances H. Arnold

- California Institute of Technology
  - Pasadena, CA
- Dick and Barbara Dickinson Professor of Chemical Engineering and Biochemistry
- Education
  - Ph.D. (Chemical Engineering), *University of California, Berkeley* (1985)

http://www.che.caltech.edu/faculty/arnold_f/

Frances H. Arnold

- Listed research interests
  - **Directed evolution**
  - Protein engineering
  - Metabolic engineering
  - Biological circuit design
  - Biocatalysis
- On the scientific advisory board of Amyris Biotechnologies (Emeryville, CA) with Jay Keasling and others
  - Working on production of artemisinin (anti-malarial compound) through *E. coli*



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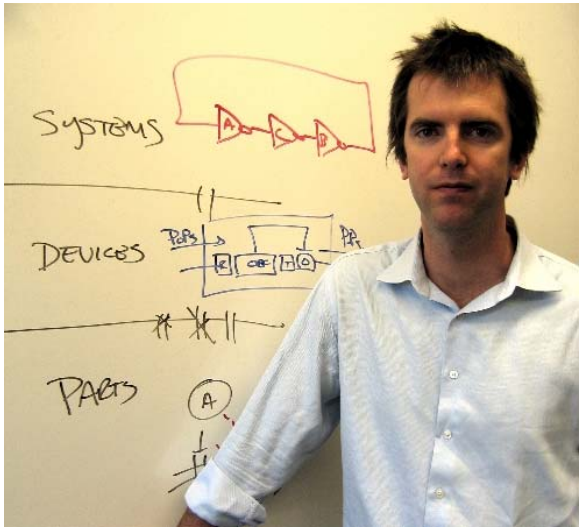
Biological Engineering – What is it?

- Metabolic engineering?
  - "The *directed* improvement of product formation or cellular properties through the modification of *specific* biochemical reactions or the introduction of new ones with the use of recombinant DNA technology." – Gregory Stephanopoulos
- Synthetic biology?
  - "For engineering, biology is a technology…synthetic biology seeks to combine a broad expansion of biotechnology applications with…an emphasis on the development of technologies that make the design and construction of engineered biological systems easier." – Drew Endy



Stephanopoulos G. *Metab Eng*. 1999 Jan;1(1):1-11.
Endy D. *Nature*. 2005 Nov 24;438(7067):449-53.

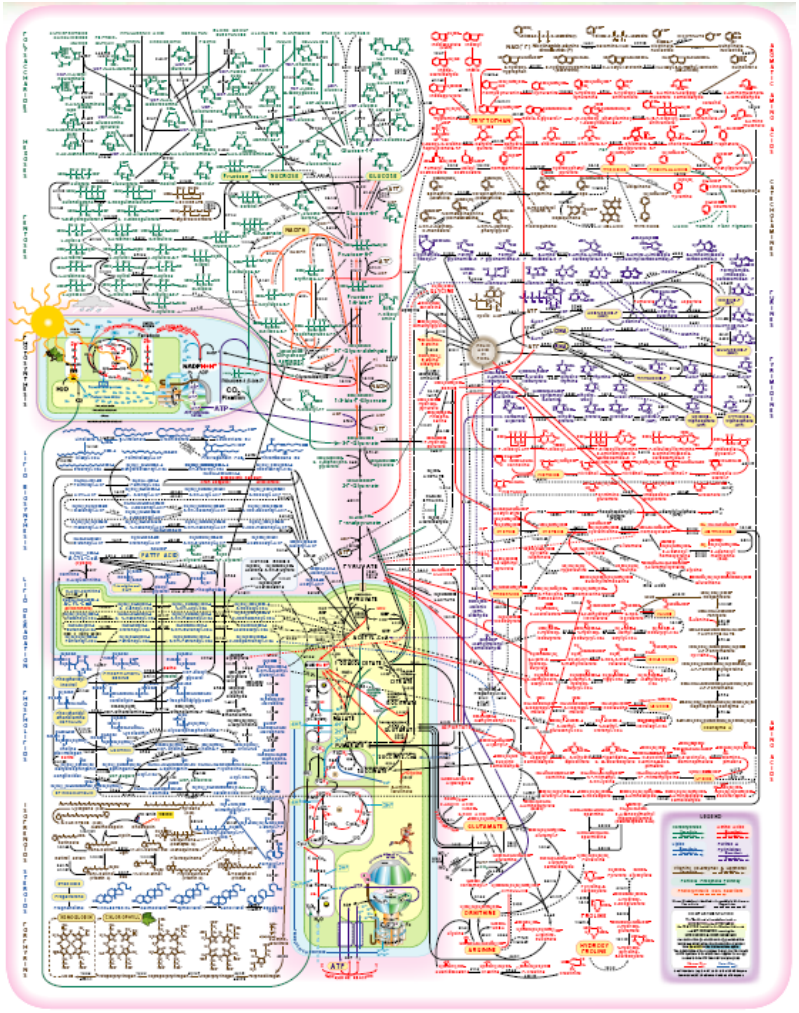
Biological Engineering – Problems

- "Why is engineering of useful synthetic biological systems still an expensive, unreliable, and *ad hoc* research process?" – DE
  - Biological systems are complex
    - Fundamentally?
    - Currently?
  - Too interdisciplinary of a field
    - Need restructuring of funding agencies
    - Need larger, but more localized research groups

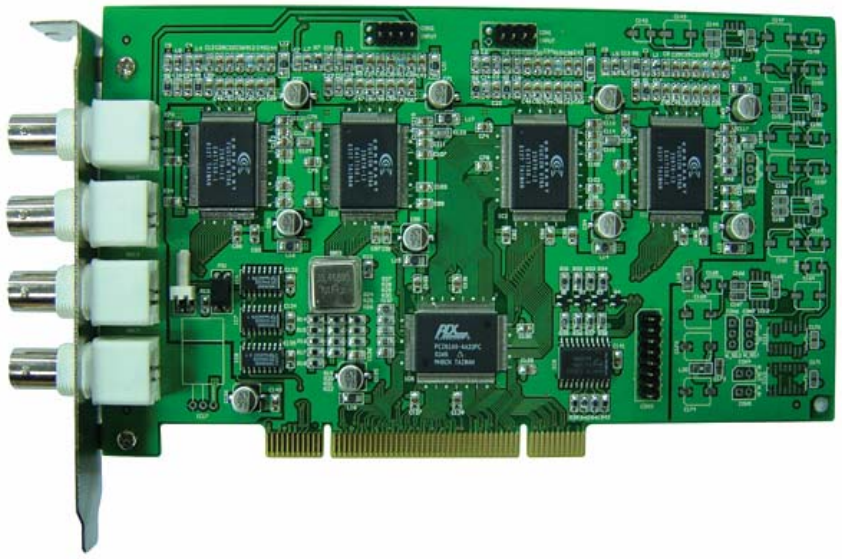
Endy D. *Nature*. 2005 Nov 24;438(7067):449-53.

Biological Engineering – Analogy

DNA (language) → protein (function)



C++ (language) → computation (function)



http://www.sigmaaldrich.com/img/assets/4202/MetabolicPathways_6_17_04_.pdf

http://img.ebigchina.com/cdimg/216530/1253913/0/1111380548.jpg

Biological Engineering – Reductionism

- Biology is (elegant) complex chemistry
- Physics is the underlying basis for all chemistry

- Engineer biological systems as if they were chemical systems

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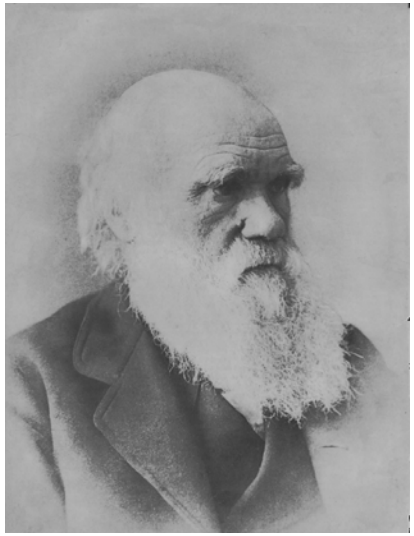
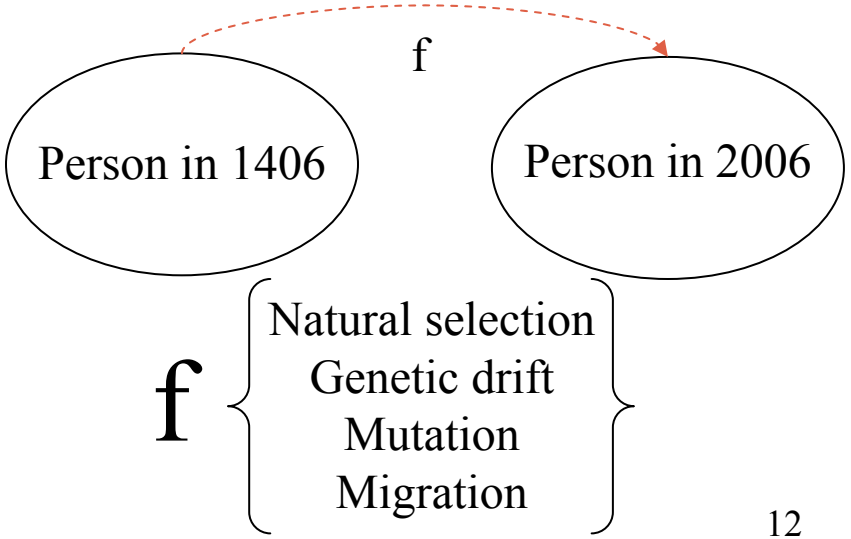
Background – Evolution

- Genotypic evolution → phenotypic evolution
- "Staunch Darwinists attribute all the complexity of living things to an algorithm of mutation and natural selection." – FHA
- **Example:** bacterial competition by antibacterial production



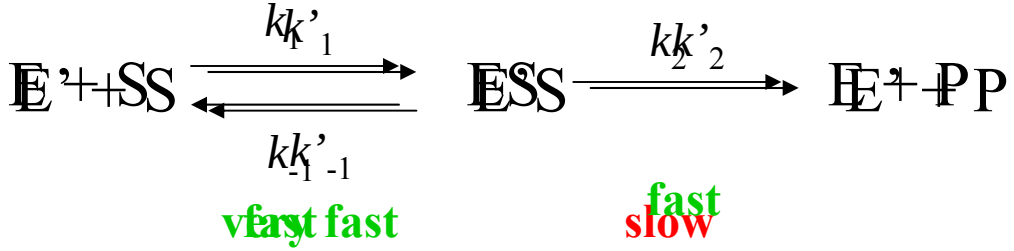


Smith GE, Dennett DC. Personal Communication.

http://www.ceha-madeira.net/ecologia/darwin.gif
http://www.che.caltech.edu/groups/fha/Enzyme/directed.html

Background – Proteins

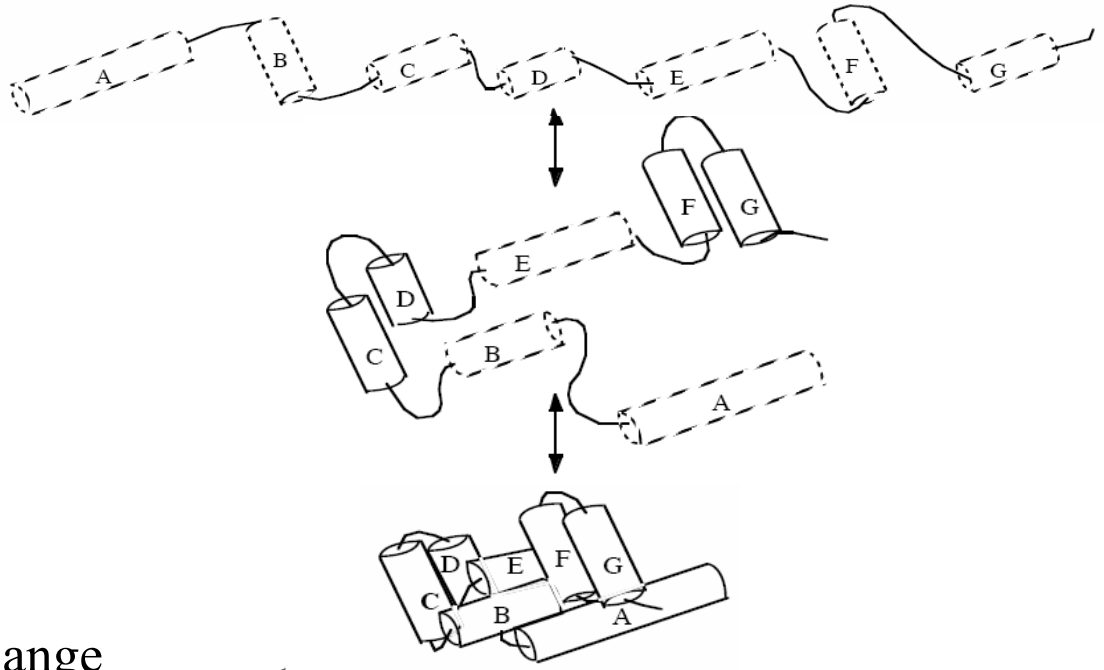
- Proteins (essentially) do everything in cells: regulation, transport, storage, structure, and catalysis (enzymes)
- Improving (evolving) proteins is important for a variety of biotechnological applications
  - Improving recombinant protein production
  - Formulating enzyme inhibitors for disease treatment

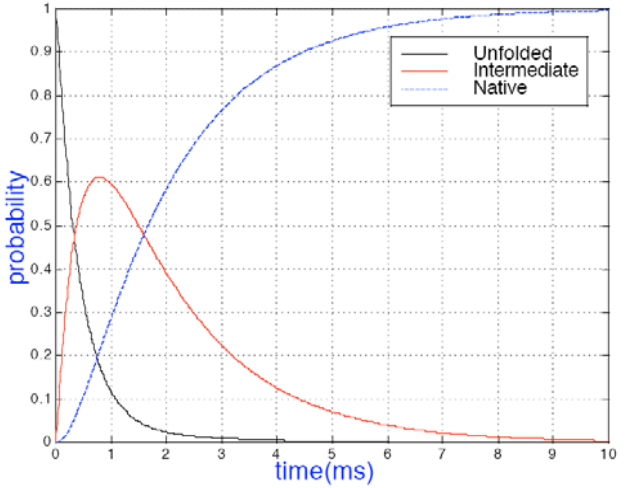


Background – Computational Biophysics

- Levinthal's Paradox
  - 100 residue protein
  - Three fundamental conformations for each residue
  - 3^{100} ($\sim 10^{48}$) possible conformations
  - 10^{-13} seconds per state-change
  - $(10^{48})(10^{-13}) = \mathbf{10^{35}}$
  - The age of the universe is approximately $\mathbf{10^{17}}$ seconds

Proteins have a preferential (functional) fold

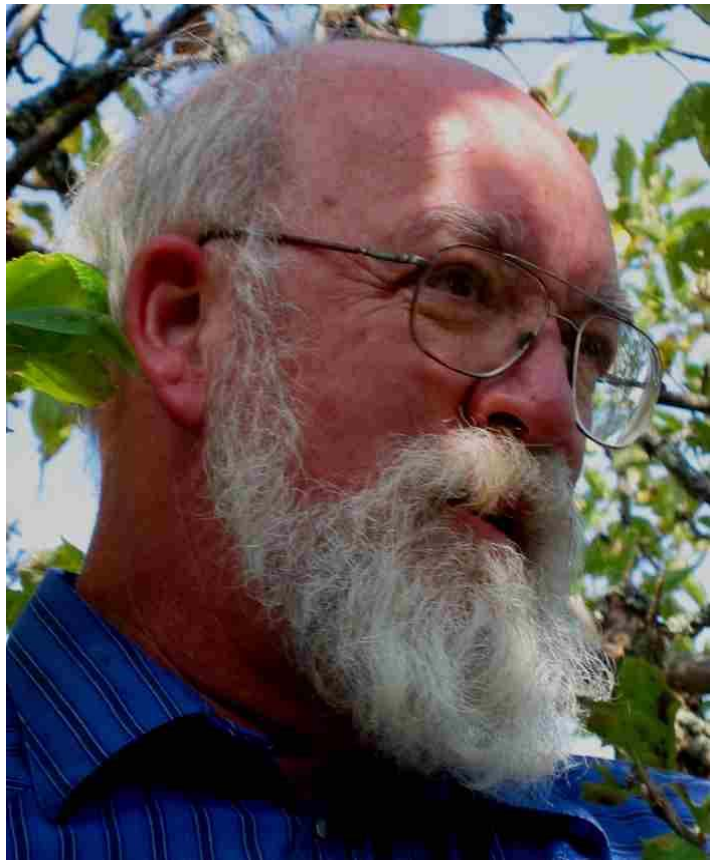




Islam SA, Karplus M, Weaver DL. *Structure*. 2004 Oct;12(10):1833-45.

Background – Computational Biophysics

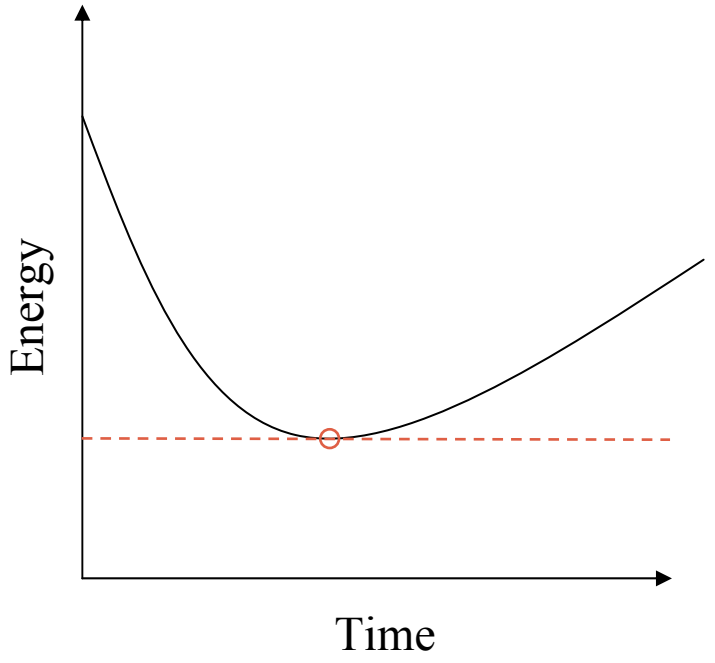
- There is a **VAST** (**V**ery much more than **AST**ronomically large) number of folds for a (rather small) protein
- What accounts for protein conformation?
  - Thermodynamics
  - Evolution



Dennett DC. *Darwin's Dangerous Idea*.
http://ase.tufts.edu/cogstud/~ddennett.htm

Background – Computational Biophysics

- Thermodynamics
  - Theoretical preferential stability
    - Hydrophobic interactions
    - Charge-charge interactions
    - Hydrogen bonding interactions
    - Disulfide covalent linkages
  - If proteins were always at their state of thermal equilibrium, they wouldn't do anything
- Evolution
  - (Supposedly) through many, many generations, our DNA has evolved to produce evolved proteins with better function

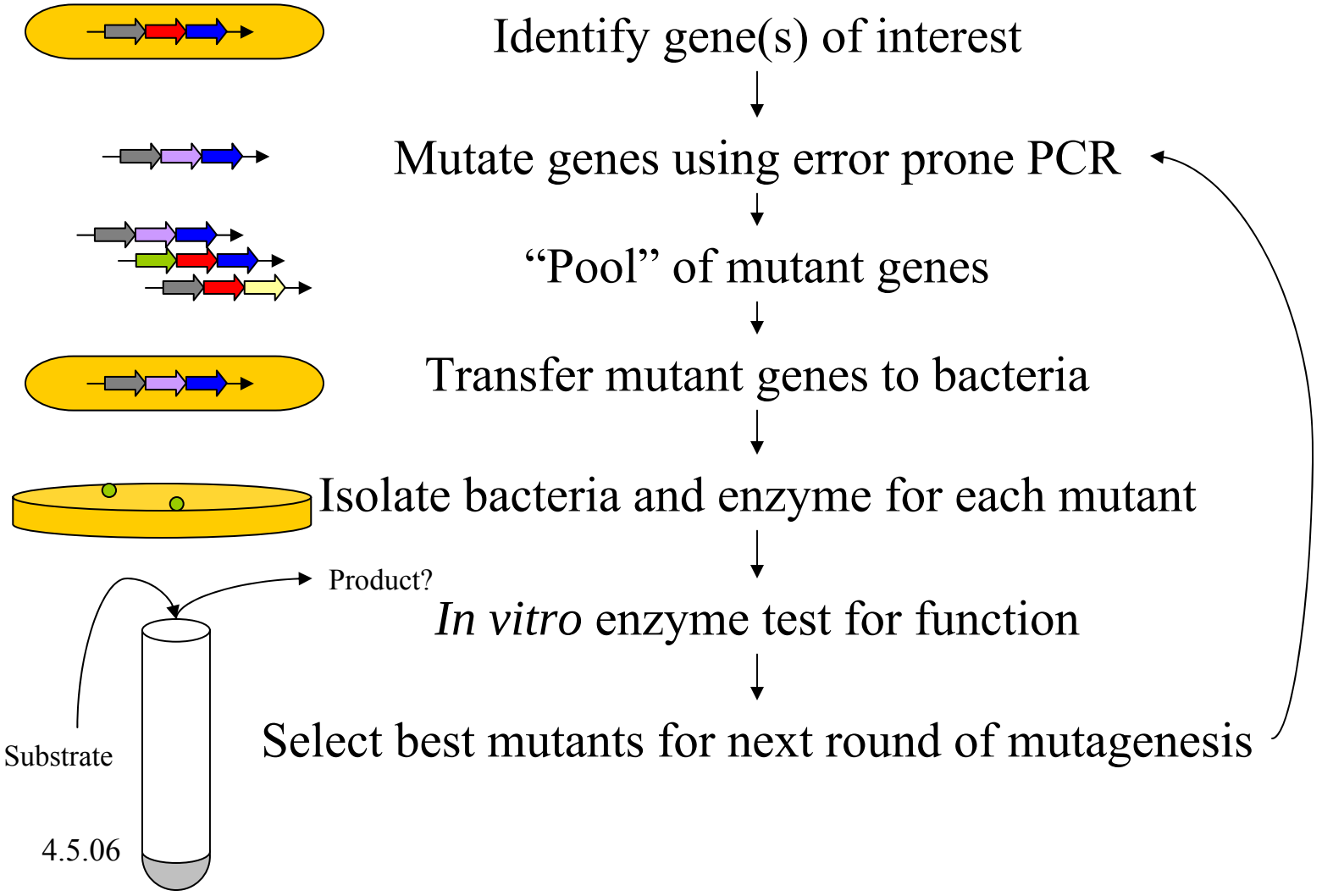


Background – Directed Evolution

We don't have the enzymes to convert ethane to ethanol ⟷ We don't need to convert ethane to ethanol to live

- To harness "evolutionary power" to create new functions for enzymes, we need to **induce mutation**
- "Directed evolution allows us to explore enzyme functions never required in the natural environment and for which the molecular basis is poorly understood." – FHA

Background – Directed Evolution



Directed Evolution – Design

- The important part is to do is in a **useful time-scale** and not an evolutionary-time scale
- Another (similar) example:
  - Take an existing 100-residue protein
  - Each residue can have one mutation on the residue next to it
  - 20^{100} possible sequences
  - VAST space → most of these sequences won't encode for a functional protein
- **Design is hard → need to use directed evolution**

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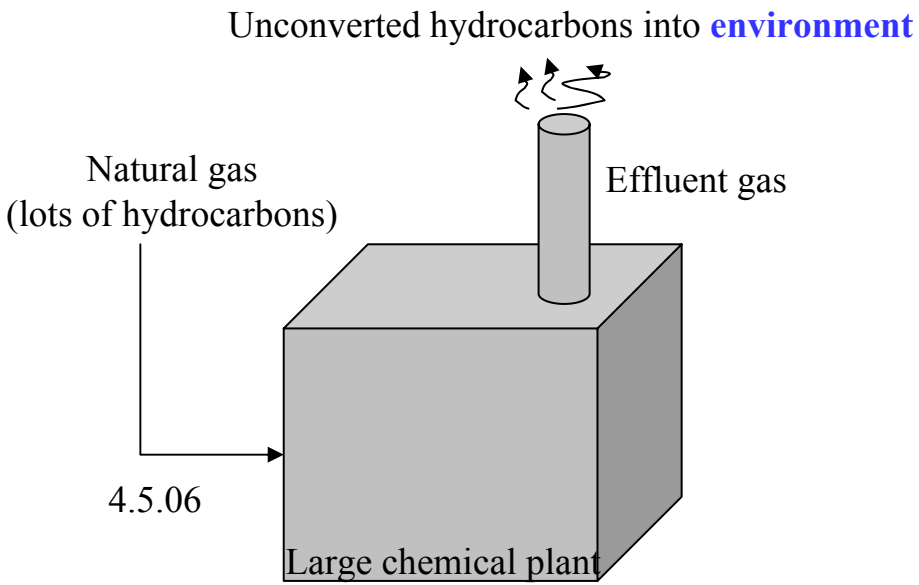
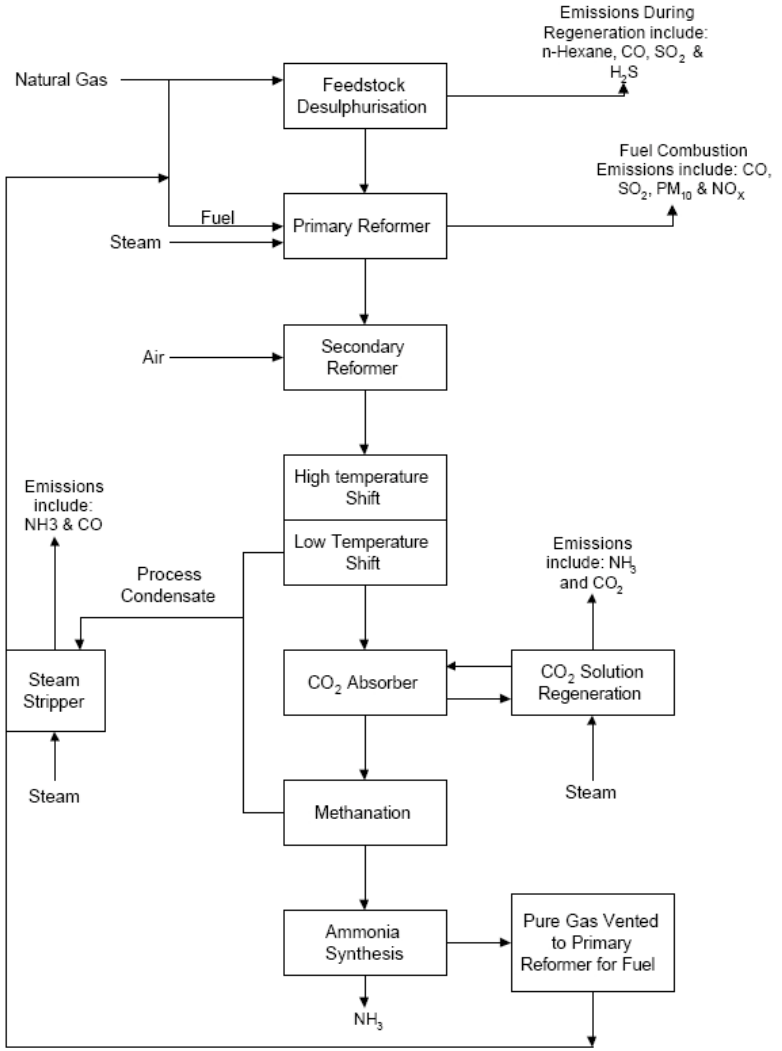
Background – Motivation Example 1

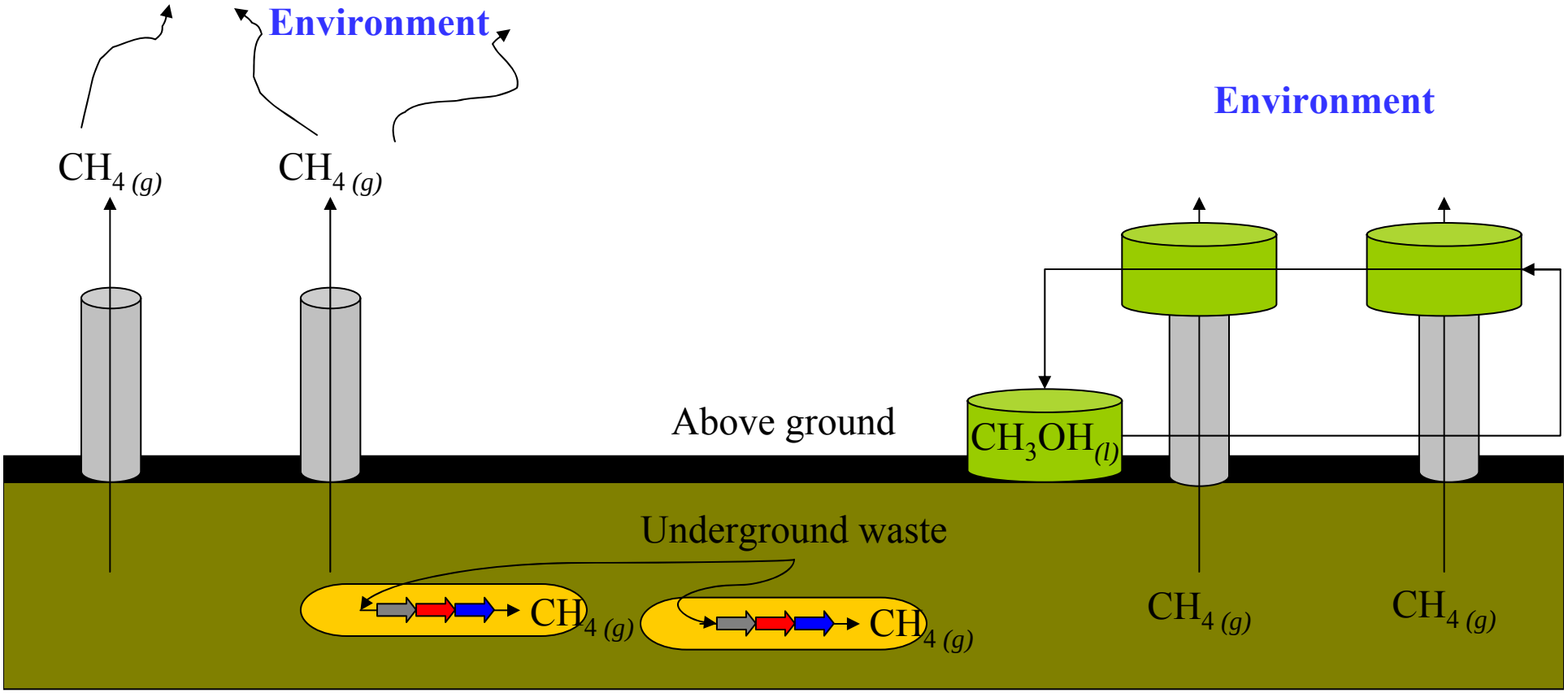
- One of the great challenges of contemporary (chemical) catalysis is the controlled oxidation of hydrocarbons
- **Example:** Haber process for ammonia production





Background – Motivation Example 2

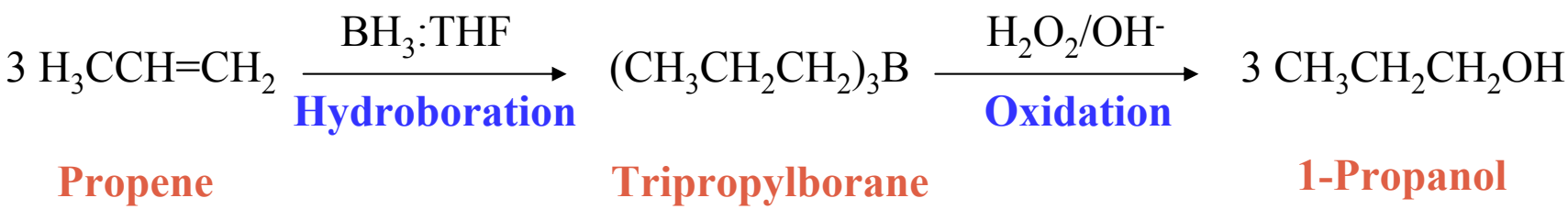
- **Example:** Trash deposit sites (dumps)



Boghigian BA. cCafferty G.

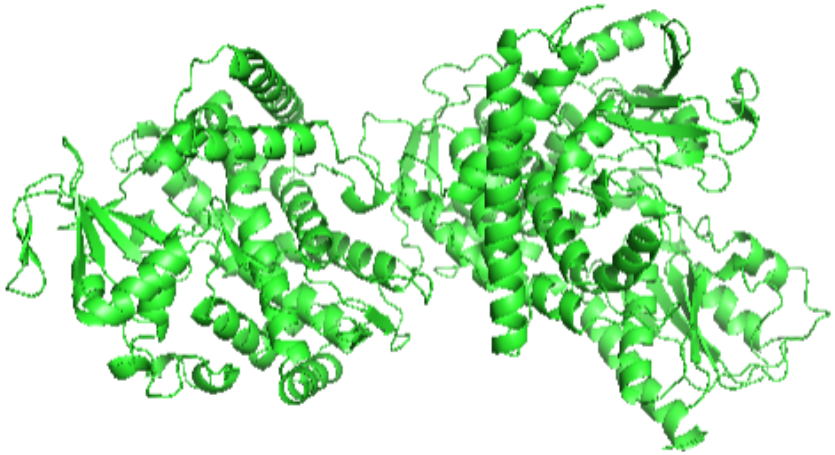
Background – Organic Chemistry

- Alkanes are the most reduced form of an organic complexes
  - Most H's possible and least O's possible
- Hydroboration-oxidation
  - Must create borane complex to oxidize to the corresponding alcohol
  - Difficult to do on a large-scale



Cytochrome P450 BM-3

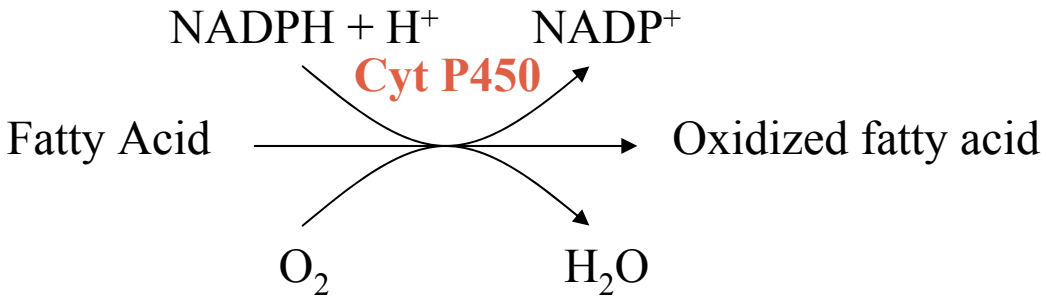
- Isolated from *Bacillus megaterium*
- EC 1.14.14.1
- Contains a hydroxylase domain (64 kDa) and a reductase domain (54 kDa) in a single polypeptide chain



Heme-domain of cytochrome P450 BM-3
(PDB ID: 1BVY)

Cytochrome P450 BM-3

- For many of the P450-catalyzed reactions, no chemical catalysts come close in performance
- Needs a substrate (fatty acid), oxygen gas, and the cofactor NADPH
- Can insert one O (from O_2) into a range of hydrophobic substrates while reducing the other O to H_2O
  - Hydroxylates fatty acids (C_{12} through C_{18})
  - Can also hydroxylate corresponding alcohols and amides



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Previous Work

- Monitor hydroxylation of octane analog spectrophotometrically for high-throughput screening

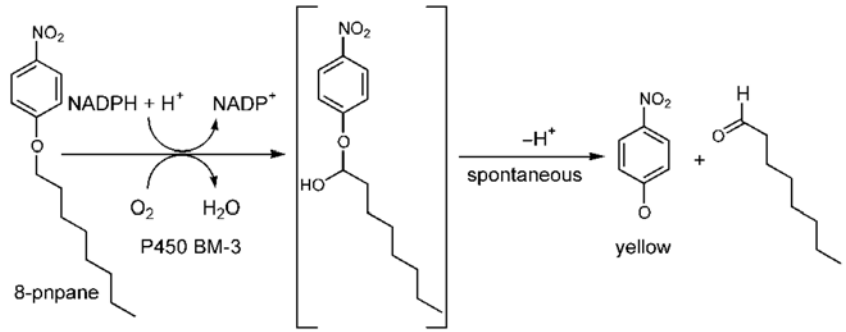


Figure 2. The screening assay for alkane oxidation activity uses the substrate analogue 8-pnpane. Terminal hydroxylation generates the unstable hemiacetal, which decomposes to the aldehyde and *p*-nitrophenolate, which is monitored at 410 nm.

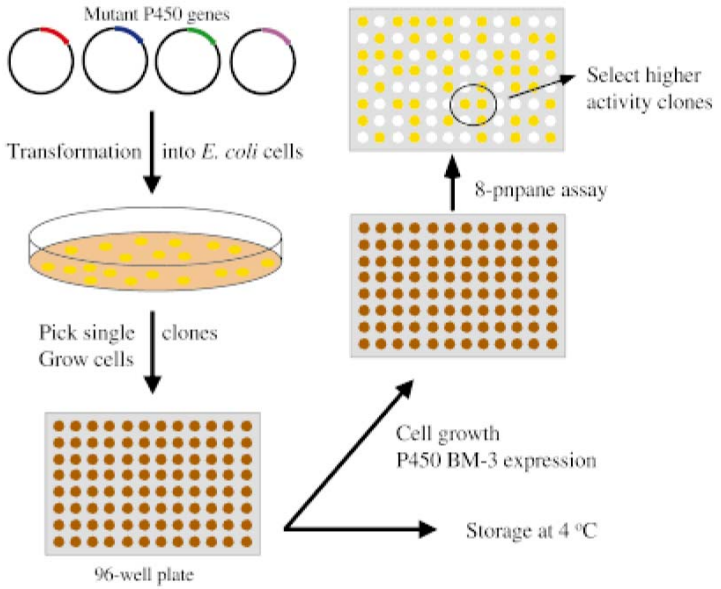
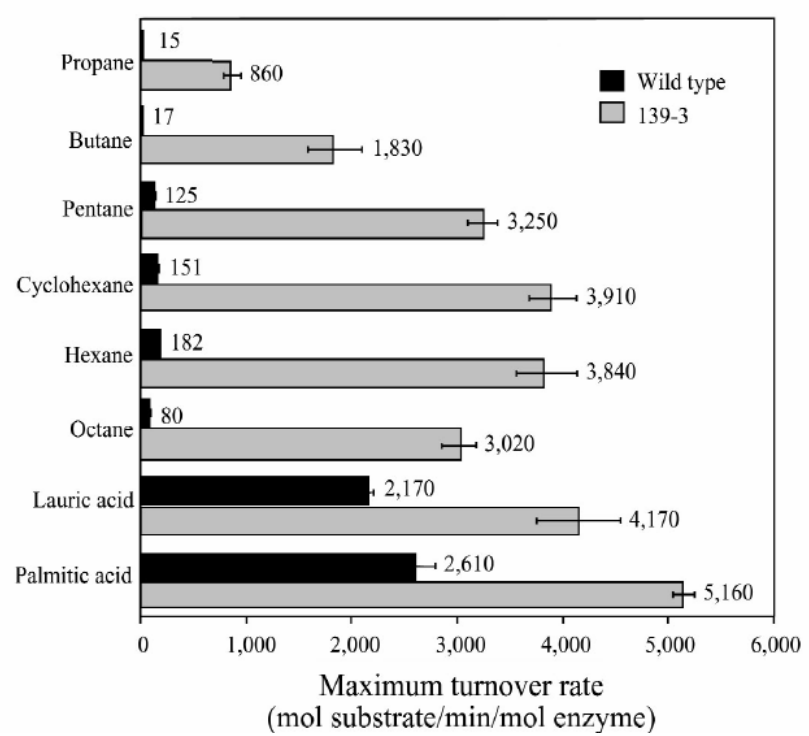


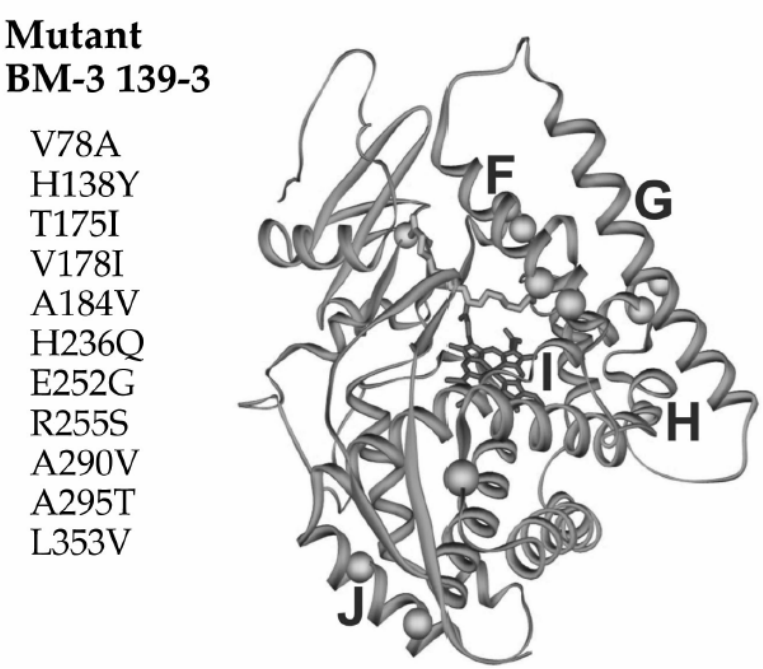
Figure 3. P450 BM-3 screening procedure. Library of P450 BM-3 mutant genes is transformed into *E. coli* and plated on agar, from which single colonies are picked into 96-well plates and grown overnight. From these plates, samples are taken to inoculate fresh 96-well plates, in which the enzymes are expressed and assayed for hydroxylation activity. The plates from the overnight growth are stored at 4 °C and used to isolate active clones identified in the assay.

Farinas ET, *et al. Adv Synth Catal*. 2001 Aug;343(6-7):601–6.

Previous Work

- Five generations of mutagenesis on wild-type cytochrome P450 BM-3 to produce mutant 139-3
  - Eleven residue substitutions in the heme-domain of the enzyme





Glieder A, *et al. Nat Biotechnol*. 2002 Nov;20(11):1135−9.

Objectives

- **ULTIMATE GOAL:** evolve cytochrome P450 BM-3 for conversion of methane to methanol
- **GOAL:** continue to evolve cytochrome P450 BM-3 (now cytochrome P450 BM-3 139-9) on the heme-domain and now also the reductase domain for conversion of smaller alkanes to their corresponding alcohols

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Cytochrome P450 BM-3 Mutants

Natural Evolution

- (Wild-type)
- 139-9
- 9-10A
- 53-3H
- 35-E11

Directed Evolution

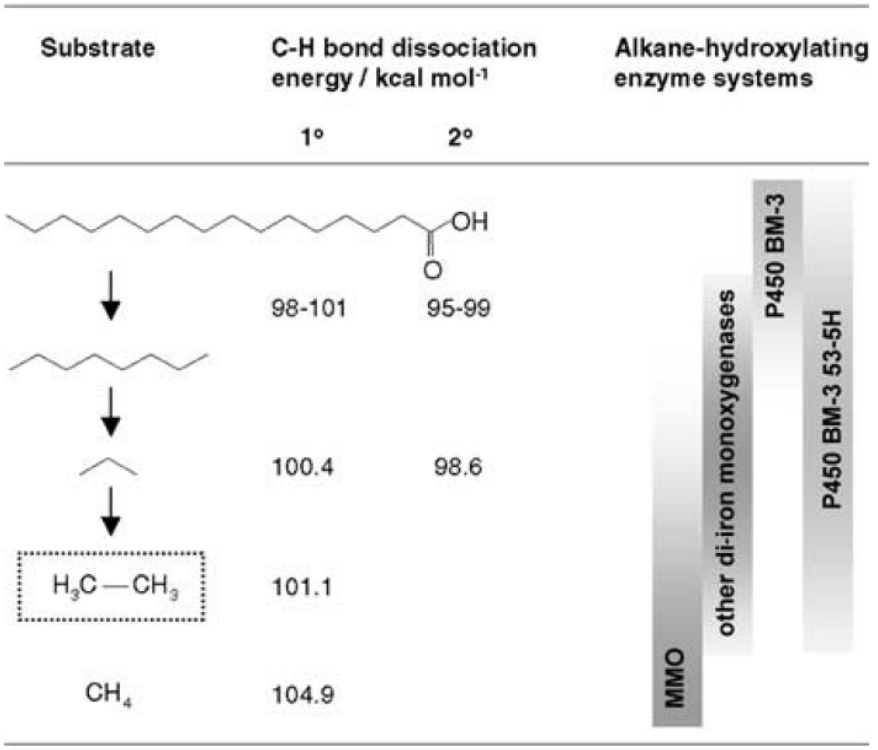

Figure 1. Variants of P450 BM-3 exhibit activities towards smaller alkane substrates, which are characterized by higher C–H bond dissociation energies. Directed evolution was used to convert wild-type P450 BM-3 stepwise from a fatty acid hydroxylase into an enzyme capable of activating the C–H bond of ethane. The figure shows the substrate range of wild-type P450 BM-3, variant 53-5H, and, for comparison, the range of substrates of other naturally occurring alkane monooxygenases.

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765–8.

P450 Expression

- Constructed plasmids (parent plasmid pT-USC1BM3) with genes encoding for P450 mutants (53-5H and 35-E11)
  - All contained 6xHis tags for purification
  - All were under the control of a P_RP_L-promoter (temperature inducible)
- Transformed into *E. coli*
- Grown in LB
  - 37°C and 250rpm until $OD_{578nm} \approx 1$
  - Induced by temperature shift to 42°C
  - Total of five hours

P450 Purification

- Cell pellet washed with Tris-HCl
- Lysed by sonication
- Lysate centrifuged at 23,300*g for 1 hour
- Supernatant filtered through 0.45 μm filter with water
- Purified by anion-exchange chromatography

Construction of first recombination library

- Error-prone PCR
  - Mutations are deliberately introduced during PCR through the use of error-prone polymerases and reaction conditions
- Randomized DNA sequences are cloned into expression vectors and the resulting mutant libraries are screened

Primers

74NNNfor (5'-GTCAA**NNN**CTTAAATTTGCACG-3')
75NNNfor (5'-GTCAAGCG**NNN**AAATTTGCACG-3')
78NNNfor (5'-GCTTAAATTT**NNN**CGTGATTTTGCAGG-3')
81NNNfor (5'-CGTGAT**NNN**GCAGGAGAC-3')
82NNNfor (5'-CGTGATTTT**NNN**GGAGAC-3')
87NNNfor (5'-GAGACGGGTTA**NNN**ACAAGCTGGAC-3')
88NNNfor (5'-GGAGACGGGTTATTT**NNN**AGCTGGACG-3')
260NNNfor (5'-CAAATTATT**NNN**TTCTTAATTGCGGGAC-3')
263NNNfor (5'-ACATTCTTA**NNN**GCGGGACACGAAAC-3')
264NNNfor (5'-ACATTCTTAATT**NNN**GGACACGAAAC-3')
328NNNfor (5'-CCAACT**NNN**CCTGCGTTTTCC-3')

NNN = mutation site

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765−8. Supplementary Information.

Construction of second recombination library

- Next, employ random mutagenesis on the reductase-domain rather than the heme-domain
- This is more difficult:
  - Less insight from crystal structure
  - Not available because of large conformational changes during catalysis

78A/T 82A/T/S/F/I/C/G/L/Vfor*
(5'-GCTTAAATTC**RCG**CGTGATTTT**DBC**GGAGACG),
78F 82A/T/S/F/I/C/G/L/V for (5'-GCTTAAATTC**TTT**CGTGATTTT**DBC**GGAGACG).
260Tfor* (5'-CAAATTATT**ACA**TTCTTAATTGCGGGAC),
260Nfor (5'-CAAATTATT**AAC**TTCTTAATTGCGGGAC),
260Lfor (5'-CAAATTATT**CTT**TTCTTAATTGCGGGAC),
87F/I/V/L 88Tfor* (5'-GAGACGGGTTA**NTYACA**AGCTGGAC),
87F/I/V/L 88Cfor (5'-GAGACGGGTTA**NTYTGT**AGCTGGAC),
328Afor* (5'-CCAACT**GCT**CCTGCGTTTTCC),
328L/F/Vfor (5'-CCAACT**BTT**CCTGCGTTTTCC),
328Mfor (5'-CCAACT**ATG**CCTGCGTTTTCC),
75L/Ifor* (5'-CTTAAGTCAAGCG**MTT**AAATTC),
75Wfor (5'-CTTAAGTCAAGCG**TGG**AAATTC).

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765−8. Supplementary Information.

Alkane Reactions

- Reactions on octane and propane were conducted as before
- Ethane reaction
  - 100, 200, or 500 nM enzyme
  - 5.0 mL, 0.1 M potassium phosphate buffer (pH 8.0)
  - Saturated with ethane and oxygen gas in a 20mL glass vial
  - 300 uL regeneration system
    - 1.66 mM NAPD+
    - 167 U/mL isocitric dehydrogenase
    - 416 mM isocitrate
  - P = 20 psi (about 1.35 atm)
  - T = 25°C
  - t = 12 hours

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765−8.

Product Analysis and NADPH Oxidation Rates

- GC/MS
  - Quantification of ethane and ethanol (no isomers, as in higher alkanes)
  - Five-point calibration curve
- UV/Vis Spectrophotometry
  - $\lambda = 340$ nm

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Alkane Reactions

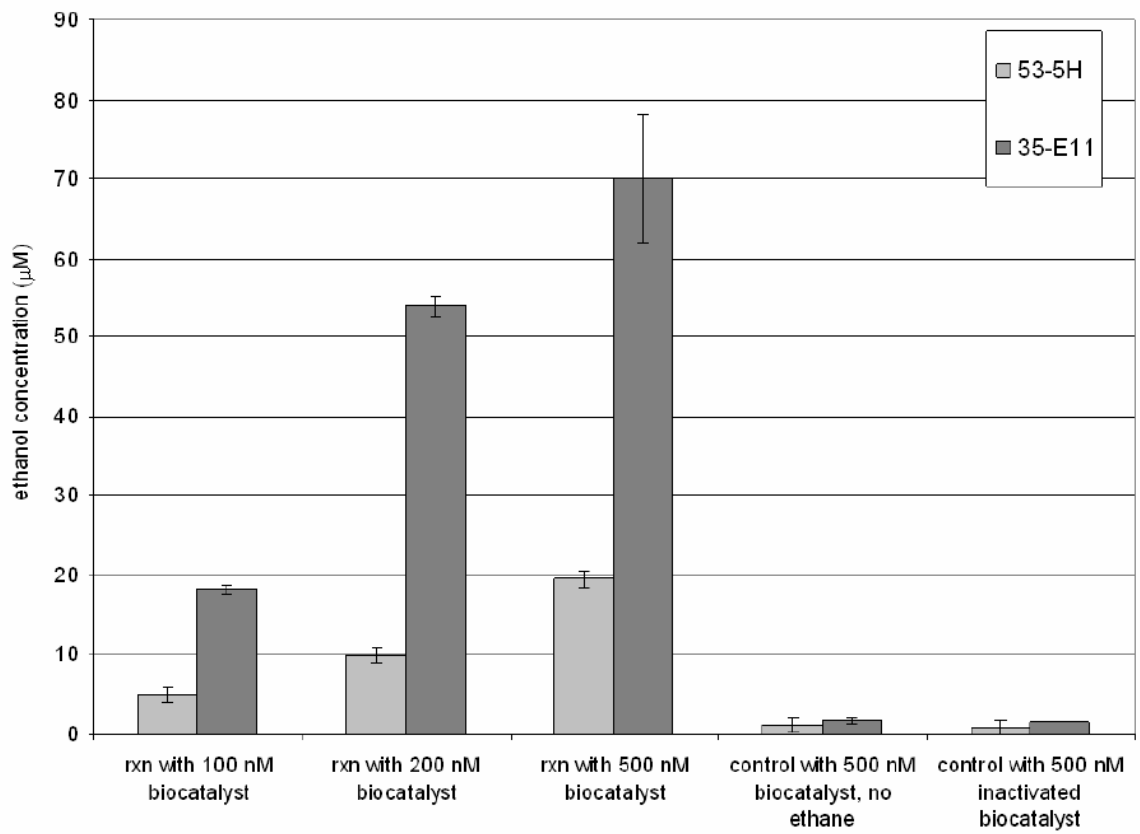

Figure S1. Final ethanol concentrations in 53-5H and 35-E11 catalyzed reactions. Conversion of ethane to ethanol using 200 nM protein corresponds to 50 and 250 turnovers per enzyme active site of 53-5H and 35-E11, respectively. Halving or doubling the enzyme concentration yielded approximately correspondingly lower or higher product concentrations, respectively. Control reactions, either without ethane or with inactivated protein, did not contain ethanol concentrations above the background level of 1 μM (1.2 ± 0.1 μM for the control reaction without ethane or 0.8 ± 0.1 μM for the control reaction with inactivated P450). Error bars are the standard deviation of three experiments.

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765−8. Supplementary Information.

GC/MS Analysis

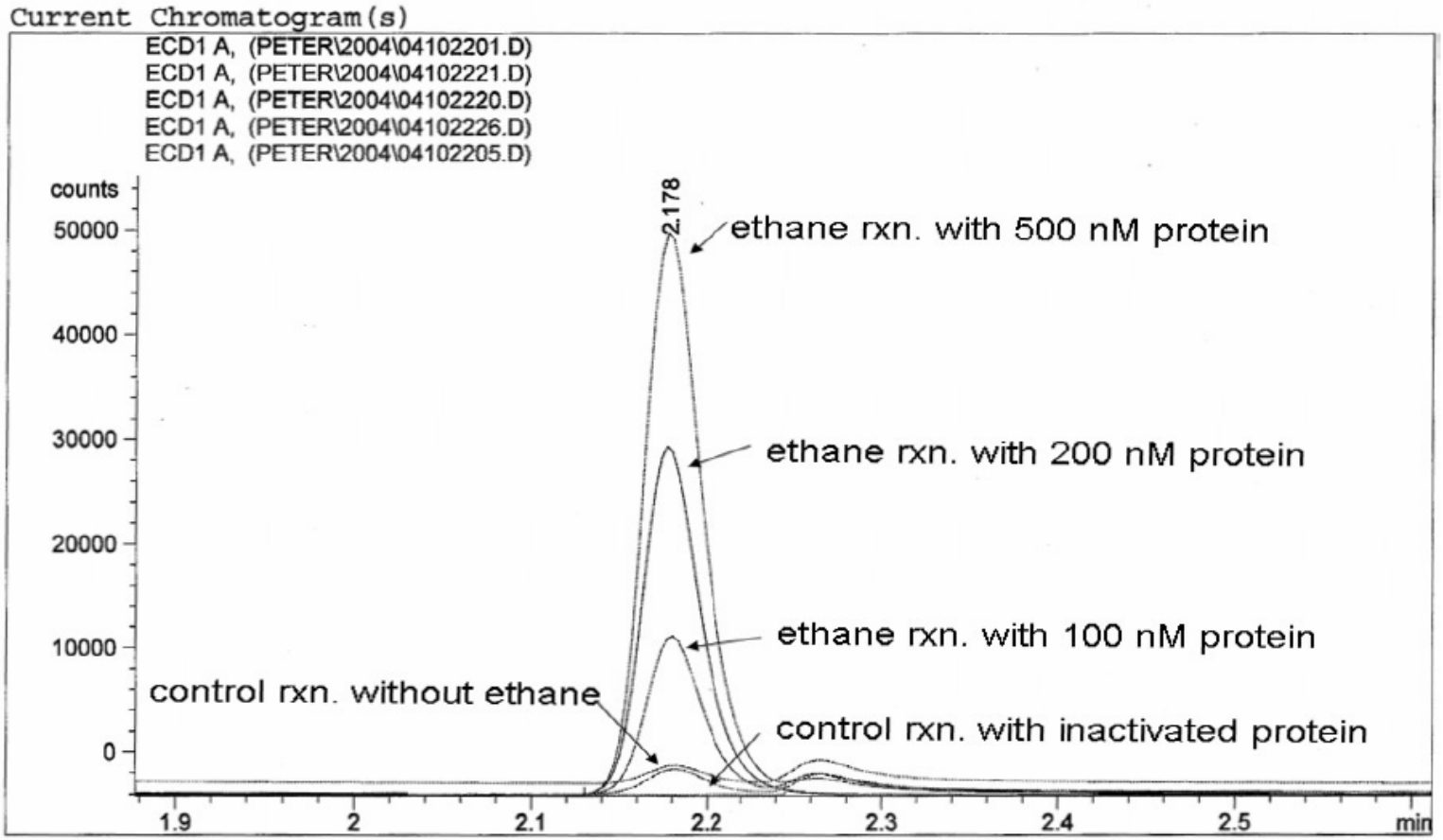

Figure S2. Gas chromatograms of ethanol reaction mixtures using variant 53-5H as the catalyst. Prior to analysis, ethanol was derivatized to form ethyl nitrite for detection via an electron capture detector (ECD).

Meinhold P, *et al. Chembiochem*. 2005 Oct;6(10):1765−8. Supplementary Information.

Summary of Evolved Enzyme Properties

Table 1. Alkane hydroxylation activities of wild-type and mutant cytochromes P450 BM-3.

Enzyme	Number of amino acid substitutions	Active site amino acid substitutions	Ethane		Propane		Octane[a]			
			Rate[b]	TTN[c]	Rate[d]	TTN[c]	Rate[d]	TTN[c]	octan-2-ol [%]	*ee* [%][e]
Wild-type BM-3	–	–	–	–	–	–	30	150	17	n.d.[f]
9-10A	13[g]	V78A	–	–	23	1100	540	3000	53	50 (*S*)
53-5H	15	V78F, A82S, A328F	0.4	50	370	5000	660	8000	89	65 (*S*)
35-E11	17	V78F, A82S, A328F	0.4	250	210	6000	420	8000	89	65 (*S*)

[a] Octane reactions were performed in the presence of 1% ethanol. [b] Rates of ethanol formation were measured over 30 min by using GC coupled to an electron-capture detector and are reported as nmol ethanol per min per nmol of enzyme. Errors are at most 10%. [c] Total turnover number (TTN) was measured by using GC after completion of the reaction and is reported as nmol product per nmol protein. Errors are at most 10%. [d] Initial rates of propanol and octanol formation were measured over 1 min by GC and are reported as nmol product per min per nmol protein. Errors are at most 15%. [e] *ee* of octan-2-ol (main product) was measured by GC; the favored enantiomer is listed in parenthesis. [f] Wild-type P450 BM-3 primarily produces octan-3- and -4-ol. The yields were not sufficient for the determination of *ee* for wt BM-3. [g] Mutant 9-10A contains amino acid substitutions R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, and L353V.

Outline

- Background
  - Journal & Corresponding Author
  - Biological Engineering
  - Molecular Evolution & Computational Biophysics
- Motivation
- Previous Work
- Experimental Methods
- Results
- Conclusions & Discussion

Conclusions

- Directed evolution is a useful tool for improving existing enzyme properties and introducing new properties
- 101.1 kcal/mol C−H bond dissociation energy did not present a fundamental barrier
- Mutagenesis on reductase-domain has significant impact on substrate specificity

Questions for Discussion

- Will the 104.9 kcal/mol C−H bond dissociation energy present a fundamental barrier?
- Will *in vivo* enzyme activity be different?
  - In practice, will it be used *in vivo*?
  - If so, will the substrates and/or enzymes be poisonous to the heterologous host?
- Can we express MMO in *E. coli* to circumvent the need for directed evolution of cytochrome P450's?
- Can we not only use this enzyme for bioremediation, but can chemists use it for a "tool" in synthetic chemistry?

Thank You