Darwin Day 2024 Lecture
Heroes of Evolution—Frances H. Arnold
Engineering Meets Evolution

Stephen L. Gasior Ph.D.
Stephen Xootfly

February 12th, 2024
Science Circle

Frances Arnold

Director

Donna and Benjamin M. Rosen Bioengineering Center Director, Rosen Bioengineering Center, 2013-

(picture from her faculty page)

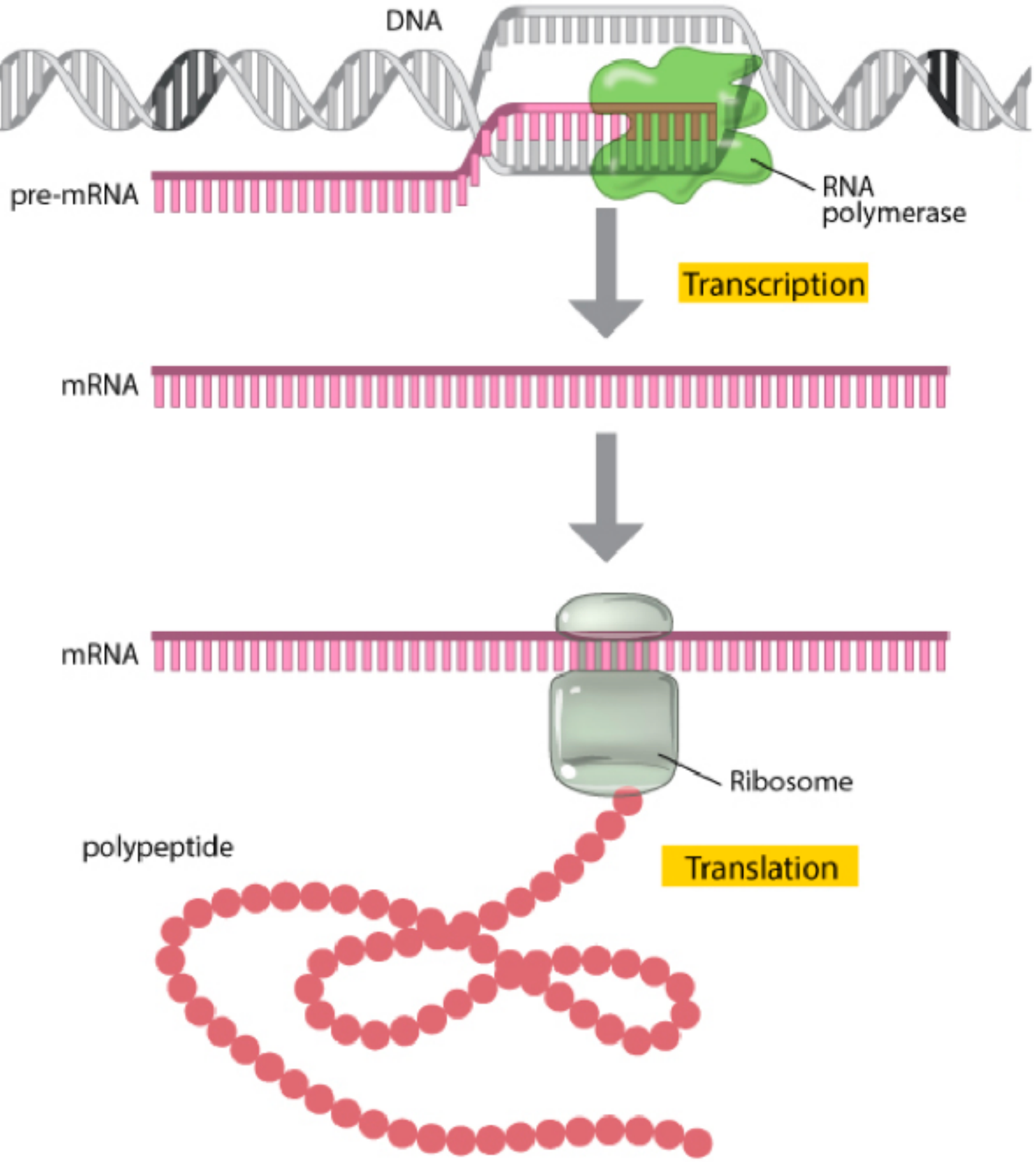
- Born born July 25, 1956
- She is the daughter of Josephine Inman (née Routheau) and nuclear physicist William Howard Arnold, and the granddaughter of Lieutenant General William Howard Arnold. Grew up in Pittsburgh suburb of Edgewood.
- As a high schooler, she hitchhiked to Washington, D.C., to protest the Vietnam War and lived on her own, working as a cocktail waitress at a local jazz club and a cab driver.
- The same independence that drove Arnold to move out of her childhood home as a teenager also led to a large volume of absences from school and low grades. In spite of this, she made near perfect scores on standardized tests and was determined to attend Princeton University
- B.S., Princeton University, 1979; Ph.D., University of California, 1985.
- Visiting Associate, Caltech, 1986; Assistant Professor of Chemical Engineering, 1987-92; Associate Professor, 1992-96; Professor, 1996-99; Professor of Chemical Engineering and Biochemistry, 1999-2000.
- Linus Pauling Professor of Chemical Engineering, Bioengineering and Biochemistry 2000-;.
- In 2018, she was co-awarded the Nobel Prize in Chemistry **"for the directed evolution of enzymes”**

nobelprize.org/.../biographical/

Arnold: Biographical Tidbits

- Father was an experimental physicist who received his PhD from Princeton in 1955 at the age of 24 and helped design the pressurized water reactor technology needed to make cheap nuclear power come true.
- In 9th grad (1969) began hitchhiking to anti-war protests in Washington DC
- Started undergrad in engineering in 1974, when the first women were graduating (Princeton only began accepting them in 1969).
- With a degree in mechanical engineering and the Carter administration's emphasis on clean, renewable energy sources, took first 'real' job (1979–1980) at a new national laboratory, the Solar Energy Research Institute (now NREL) in Golden, Colorado.
- With the election of Ronald Reagan as President of the United States, the future for passive solar heating and cooling seemed somewhat limited. … and headed west to start graduate studies at the University of California, Berkeley. Chemical engineering.
- Allan Wilson introduced her to molecular evolution of protein sequences.
- Met Jay Bailey, a world-renowned biochemical engineering professor at Caltech … and married in 1987, in Macatawa

How Enzymes are Made

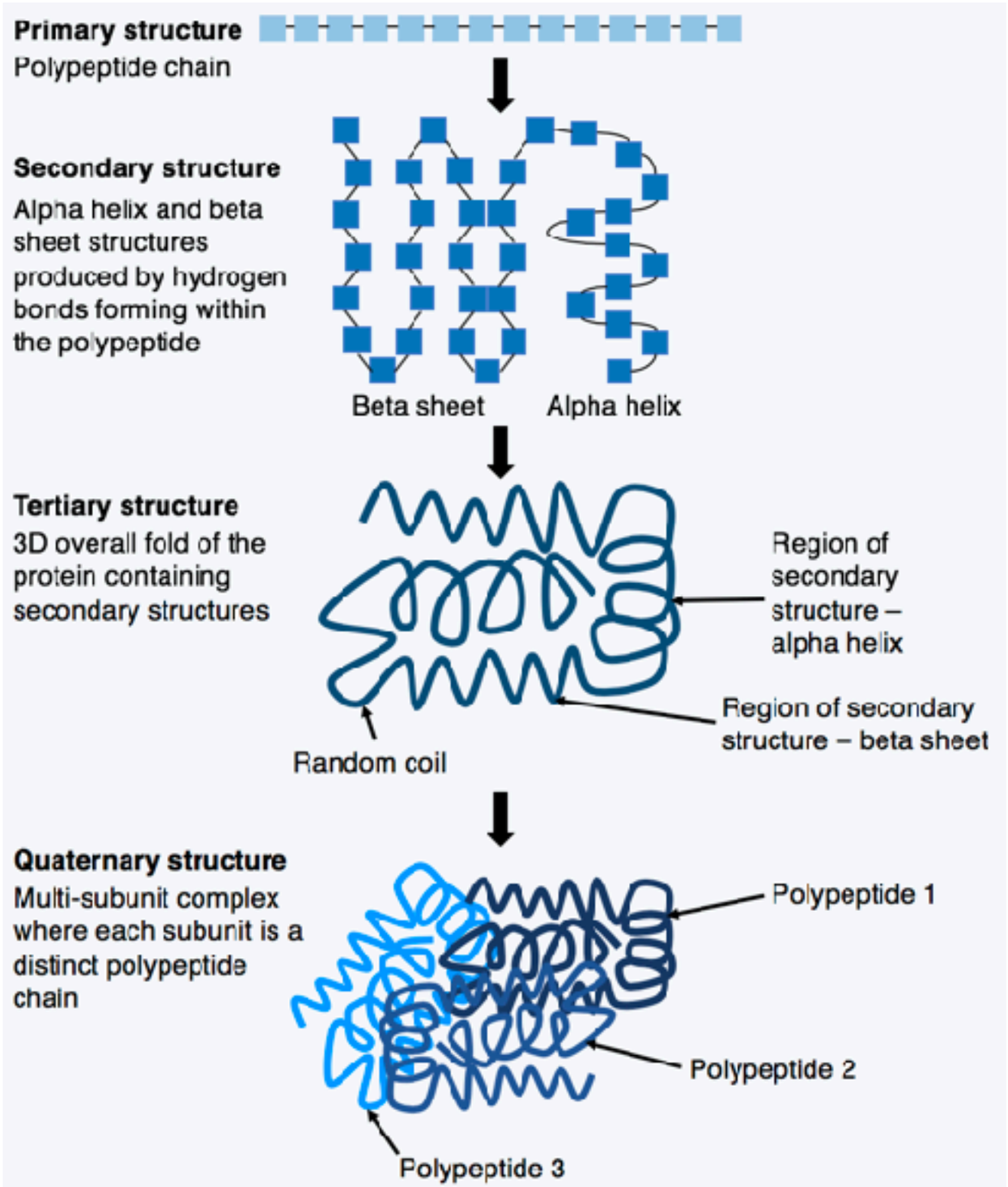


Codon Table
DNA->RNA->amino acid

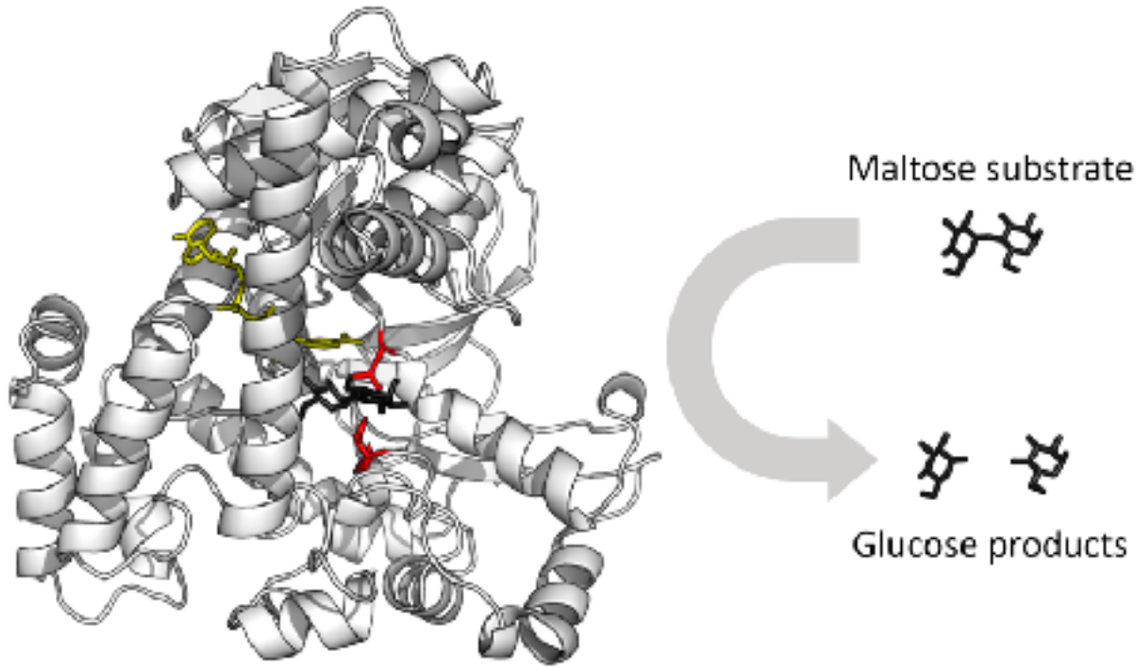
Second nucleotide

First nucleotide	U	C	A	G	Third nucleotide
U	UUU Phe UUC UUA Leu UUG	UCU UCC UCA Ser UCG	UAU Tyr UAC UAA **STOP** UAG **STOP**	UGU Cys UGC UGA **STOP** UGG Trp	U C A G
C	CUU CUC CUA Leu CUG	CCU CCC CCA Pro CCG	CAU His CAC CAA Gln CAG	CGU CGC CGA Arg CGG	U C A G
A	AUU AUC Ile AUA AUG Met	ACU ACC ACA Thr ACG	AAU Asn AAC AAA Lys AAG	AGU Ser AGC AGA Arg AGG	U C A G
G	GUU GUC GUA Val GUG	GCU GCC GCA Ala GCG	GAU Asp GAC GAA Glu GAG	GGU GGC GGA Gly GGG	U C A G

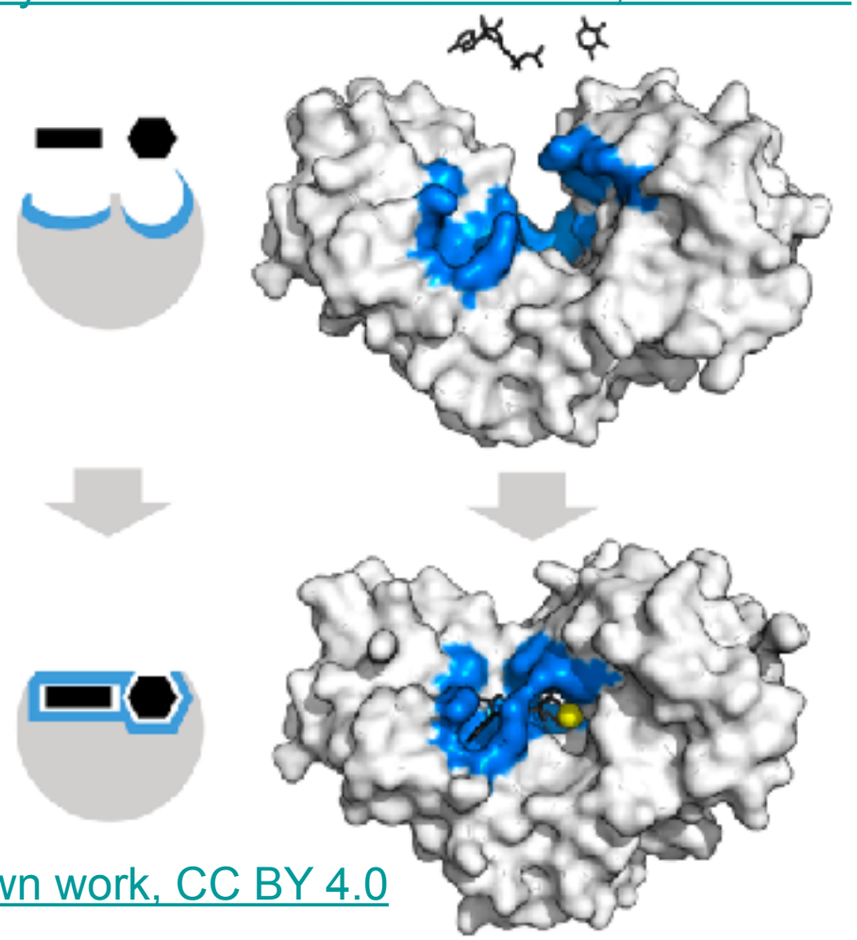
Nuts and Bolts of Enzymes: How are they Made and Do Stuff



By Kep17 - Own work, CC BY-SA 4.0

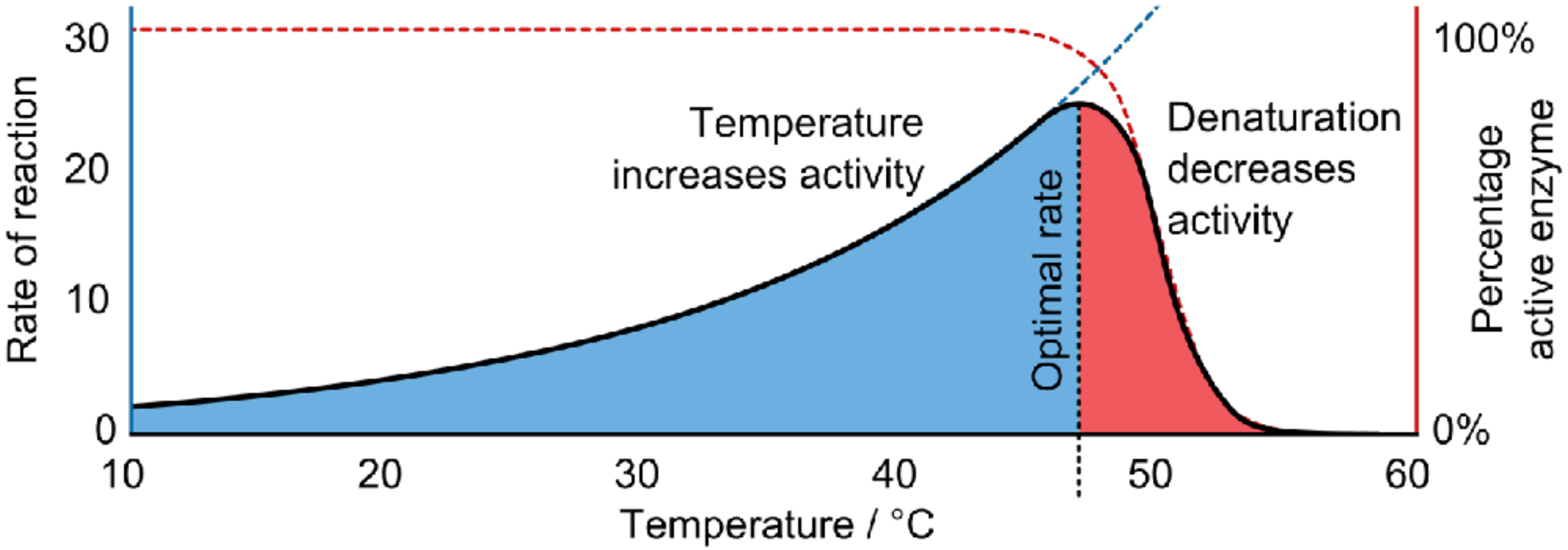


By Thomas Shafee - Own work, CC BY 4.0

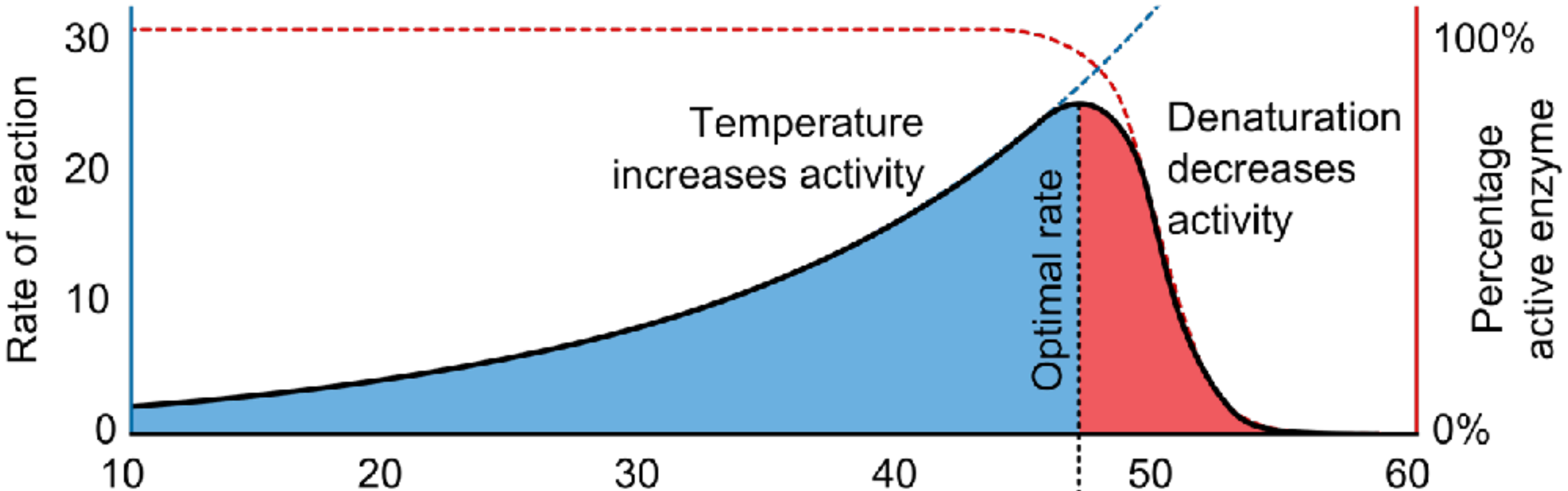


By Thomas Shafee - Own work, CC BY 4.0

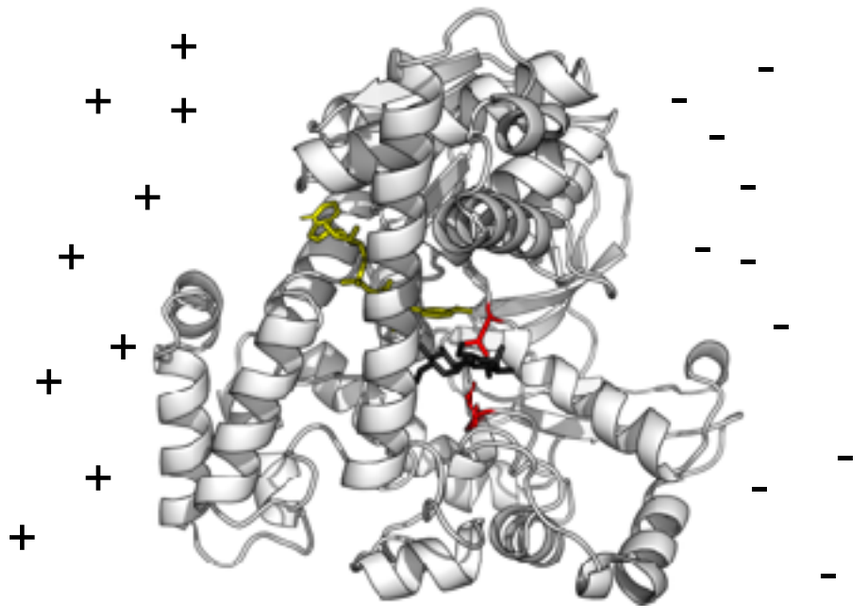
Enzyme Activity



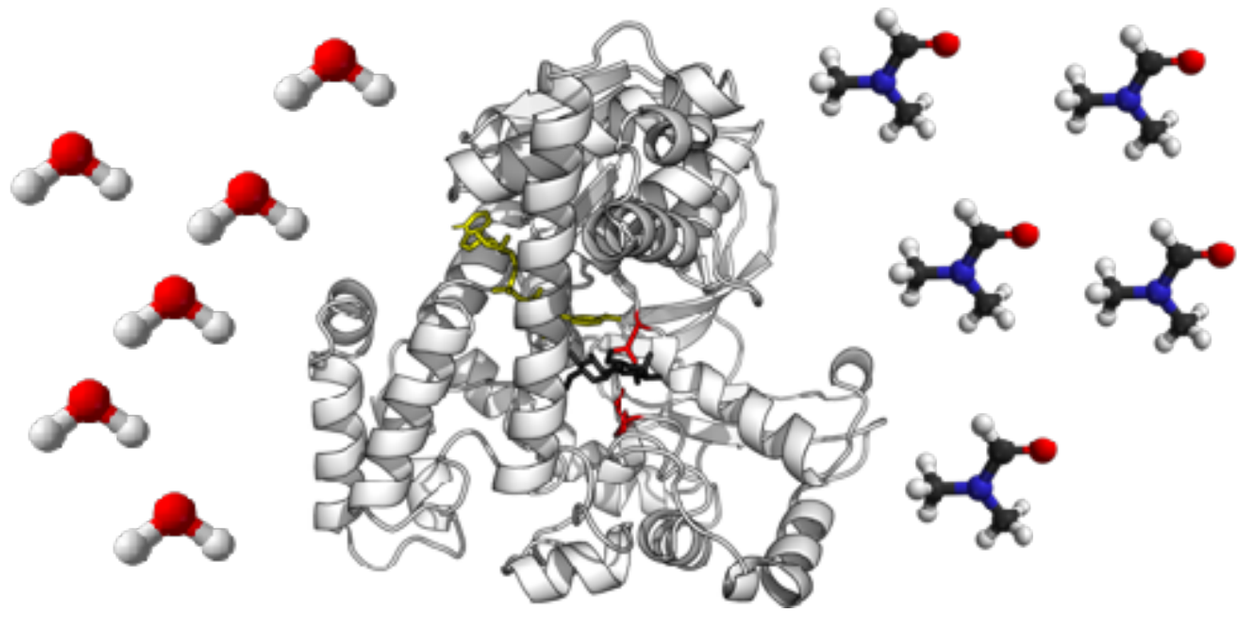
Enzyme Activity



pH of environment



aqueousness of solvent



Arnold: Directed Evolution

ENZYME ENGINEERING FOR NONAQUEOUS SOLVENTS: RANDOM MUTAGENESIS TO ENHANCE ACTIVITY OF SUBTILISIN E IN POLAR ORGANIC MEDIA

Keqin Chen and Frances H. Arnold*

Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, Pasadena, CA 91125.
*Corresponding author.

Subtilisin E expression by colonies of bacteria produce “halos” of casein processing

converts suc-Ala-Ala-Pro-Phe-p-nitroanilide (sAAAPF-pna) for quantitative data

using error-prone PCR to introduce mutations in DNA (misbalanced dATP)

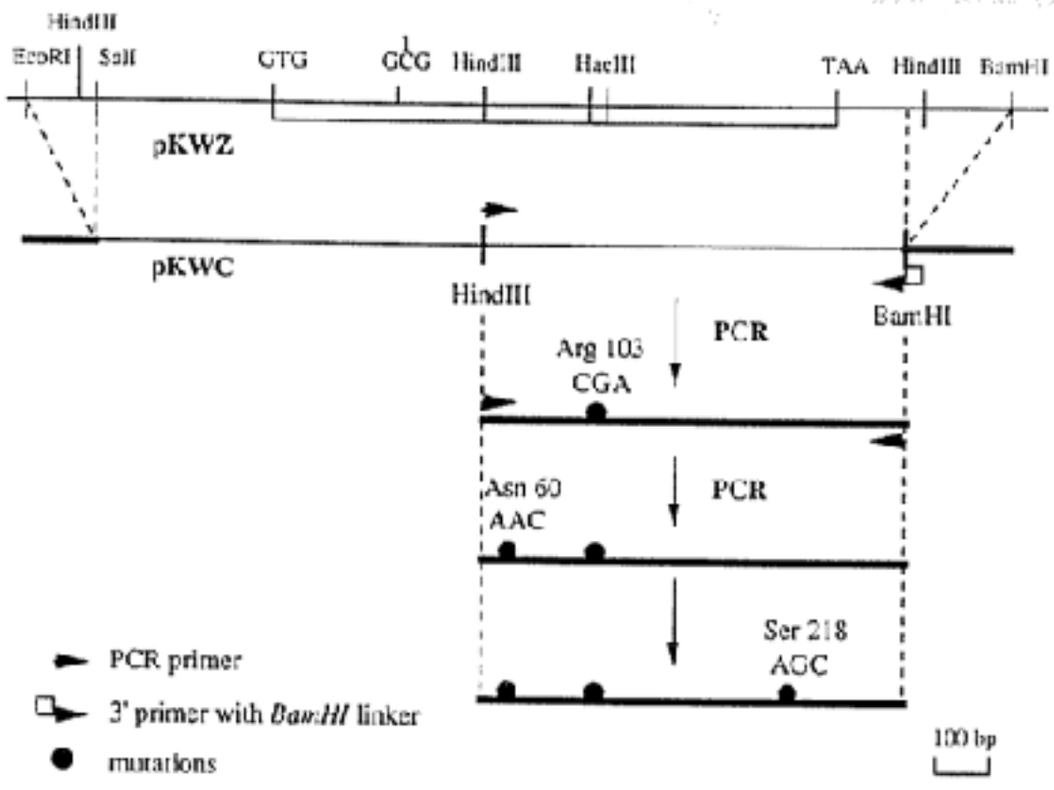


FIGURE 1 General scheme for construction of plasmid pKWC and random mutagenesis of subtilisin E, whose coding region is framed in pKWZ. Two regions of pKWZ deleted to generate pKWC are indicated by dashed lines. Base substitutions obtained after random mutagenesis by PCR and for the N218S mutation are indicated on the PCR-targeted HindIII-BamHI fragments.

Arnold: Directed Evolution

TABLE 1 Kinetic constants and incremental free energies of transition state stabilization ($\Delta\Delta G^{\ddagger}$) for hydrolysis of sAAPFpna by wild-type and variant subtilisins E. Conditions are 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 8.0 and stated amount of DMF. Errors in reported K_M and k_{cat} are ±10%. The effects of amino acid substitutions on transition state stabilization energies $\Delta\Delta G^{\ddagger}$ for the hydrolysis reaction were determined from the specificity constants k_{cat}/K_M: $\Delta\Delta G^{\ddagger} = -RT \ln (k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\text{-}type}$.

	0% DMF				10% DMF				20% DMF	
	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M $M^{-1}\,s^{-1} \times 10^{-3}$	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M $M^{-1}\,s^{-1} \times 10^{-3}$	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}	k_{cat}/K_M M^{-1} $s^{-1} \times 10^{-3}$	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}
WT	21	0.56	38	-	18	2.8	6.3	-	1.4	-
Q103R	31	0.25	124	−0.73	32	1.4	23	−0.80	6.8	−0.98
D60N	22	0.53	42	−0.06	30	3.2	9.6	−0.26	3.1	−0.51
Q103R+D60N	26	0.20	130	−0.76	43	1.2	36	−1.07	12	−1.36
D60N+N218S	37	0.46	80	−0.46	54	2.7	20	−0.71	9.2	−1.18
Q103R+D60N+N218S	40	0.11	360	−1.39	71	0.70	110	−1.76	45	−2.15

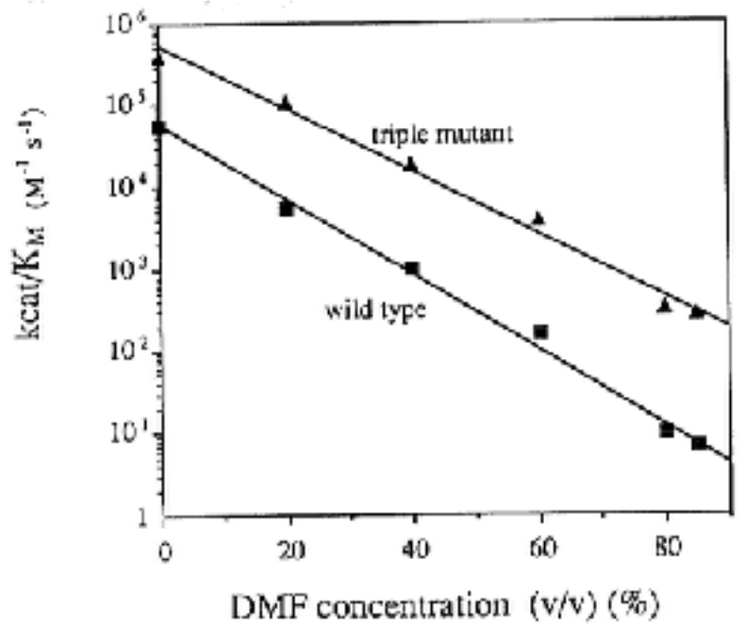


FIGURE 2 Catalytic efficiency for hydrolysis of sAAPM-pna by wild-type subtilisin E (■) and triple mutant Q103R+D60N+N218S (▲). Specificity constants k_{cat}/K_M were determined from initial rate experiments at low substrate concentration (0.01–0.1 K_M) in 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 8.0 with stated amounts (v/v %) of DMF.

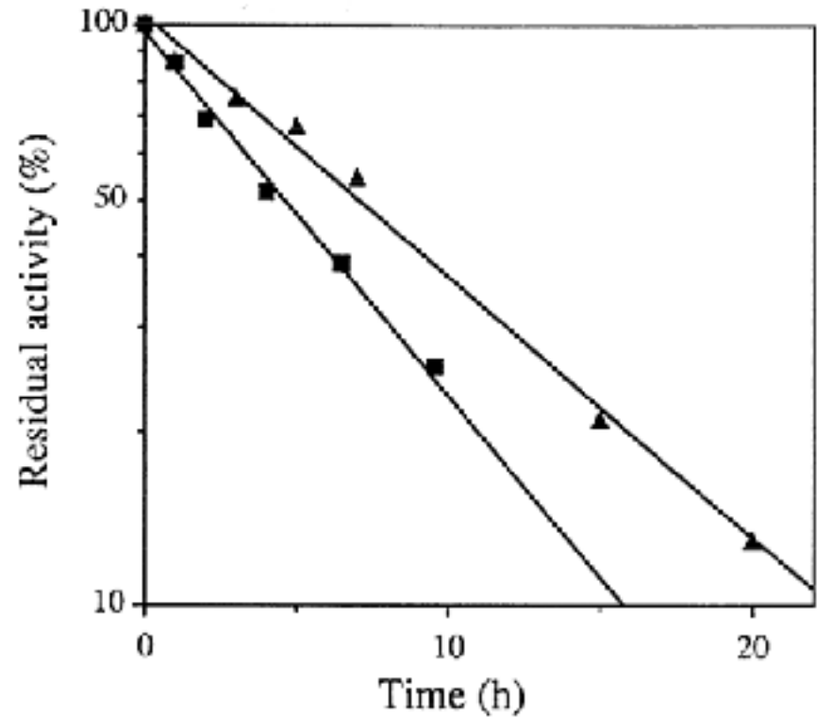
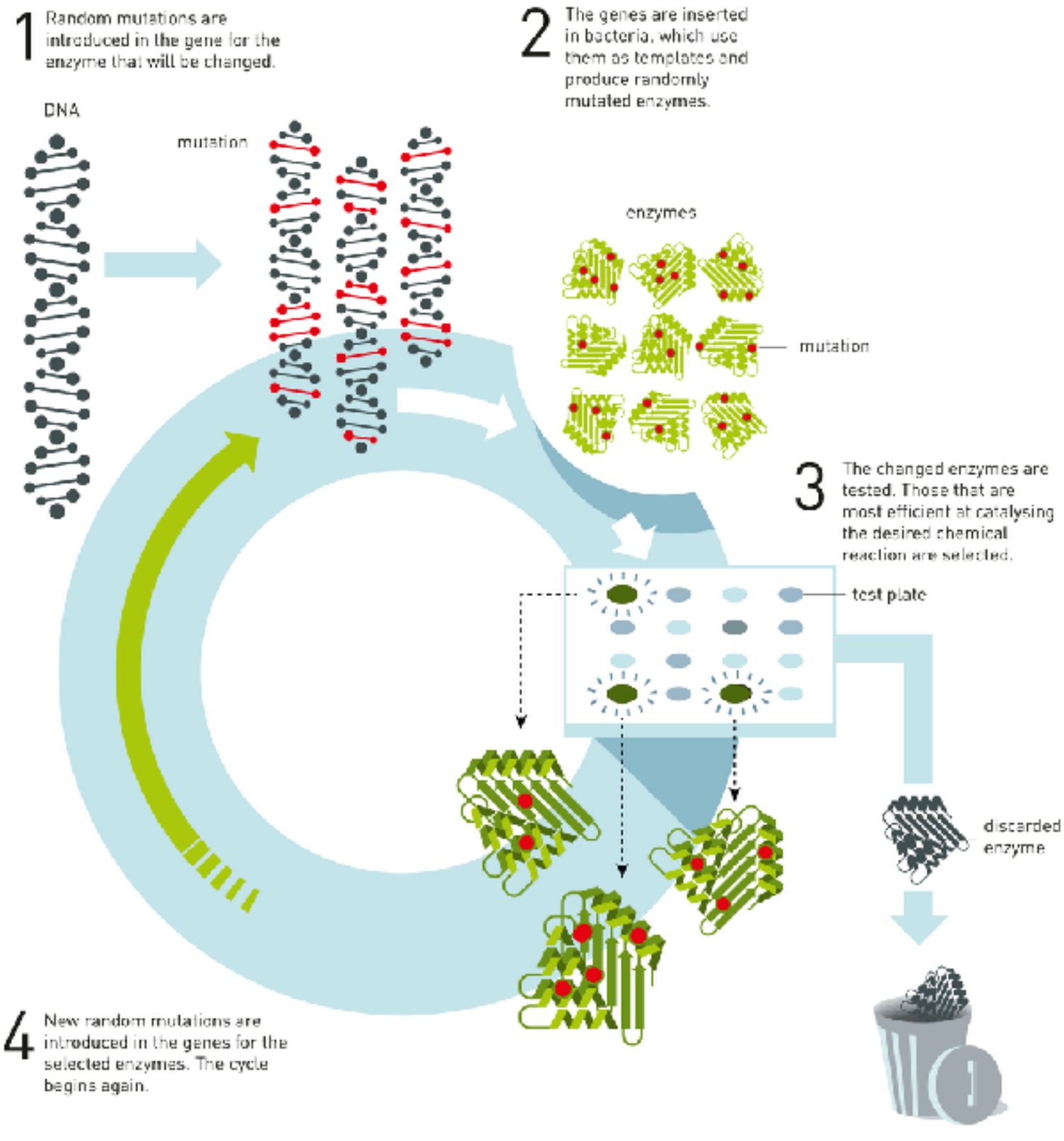


FIGURE 3 Deactivation of wild-type (■) and Q103R+D60N+N218S (▲) subtilisin E in 40% DMF, 50°C.

Arnold: Directed Evolution



Arnold: Directed Evolution

Tuning the activity of an enzyme for unusual environments: Sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide

(peptide synthesis/biocatalysis/molecular evolution/serine protease)

KEQIN CHEN AND FRANCES H. ARNOLD[1]

Sequential rounds of mutagenesis and screening have yielded a variant (PC3) that hydrolyzes a peptide substrate 256 times more efficiently than wild-type subtilisin in 60% dimethylformamide (DMF).

Starting with a variant containing four effective amino acid substitutions … found an additional 6.

PC3 subtilisin is 130 times more efficient than wild-type subtilisin E in 40% DMF.

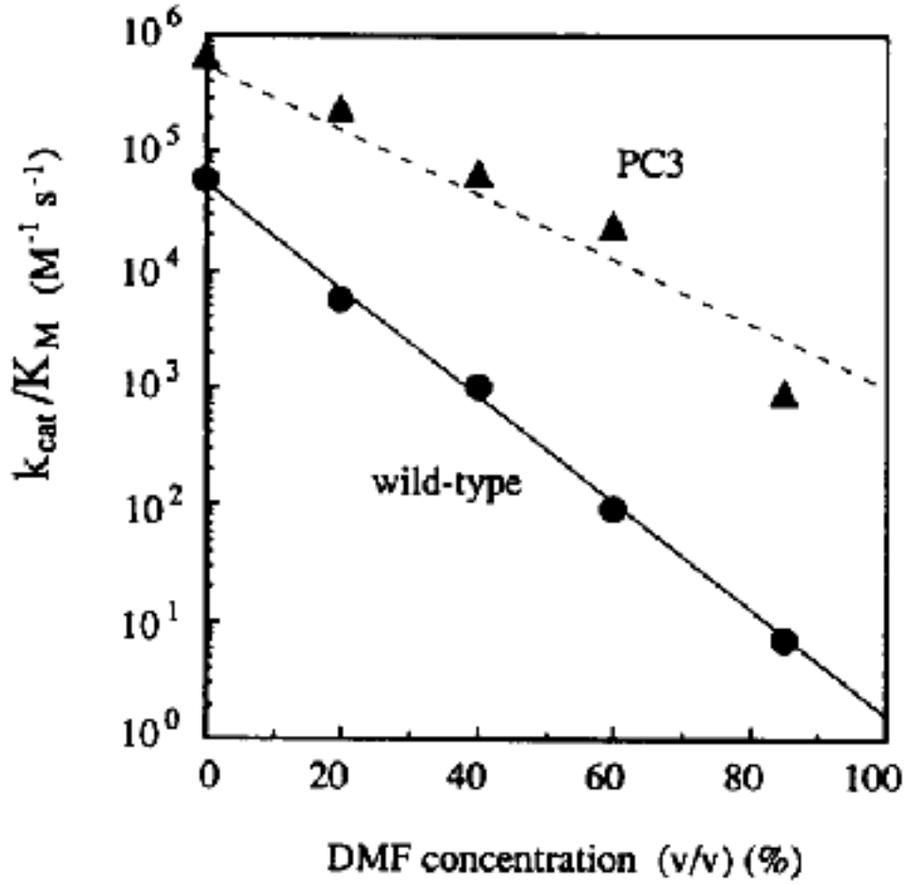


FIG. 1. Catalytic efficiency for hydrolysis of sAAPM-pna by wild-type subtilisin E (●) and PC3 (▲). k_{cat}/K_m values were determined from initial rates at low substrate concentrations in 0.1 M Tris·HCl/10 mM $CaCl_2$, pH 8.0 at 37°C and specified amounts (vol/vol) of DMF.

Arnold: Directed Evolution

Directed evolution of subtilisin E in *Bacillus subtilis* to enhance total activity in aqueous dimethylformamide

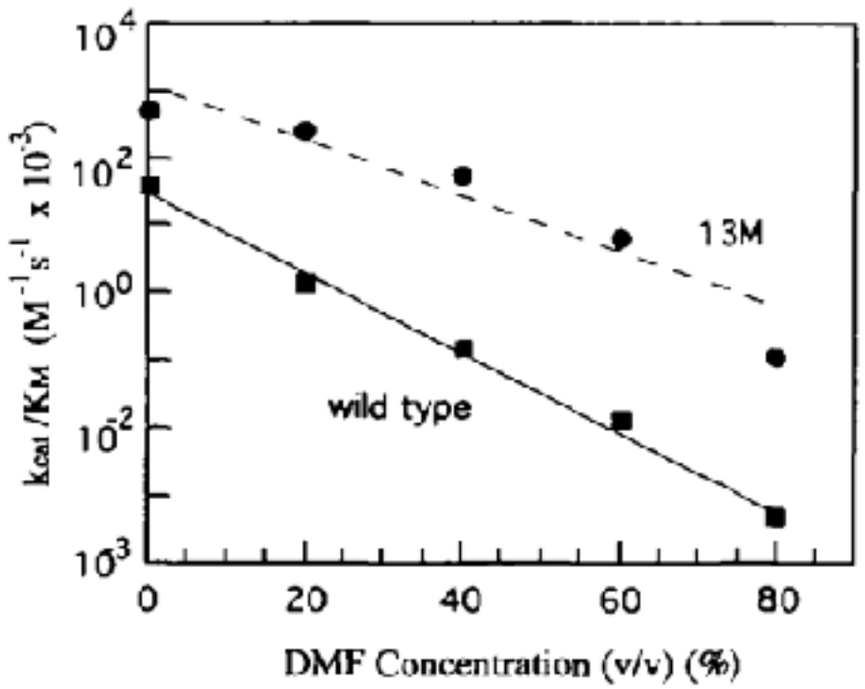
L.You[1] and F.H.Arnold[2]

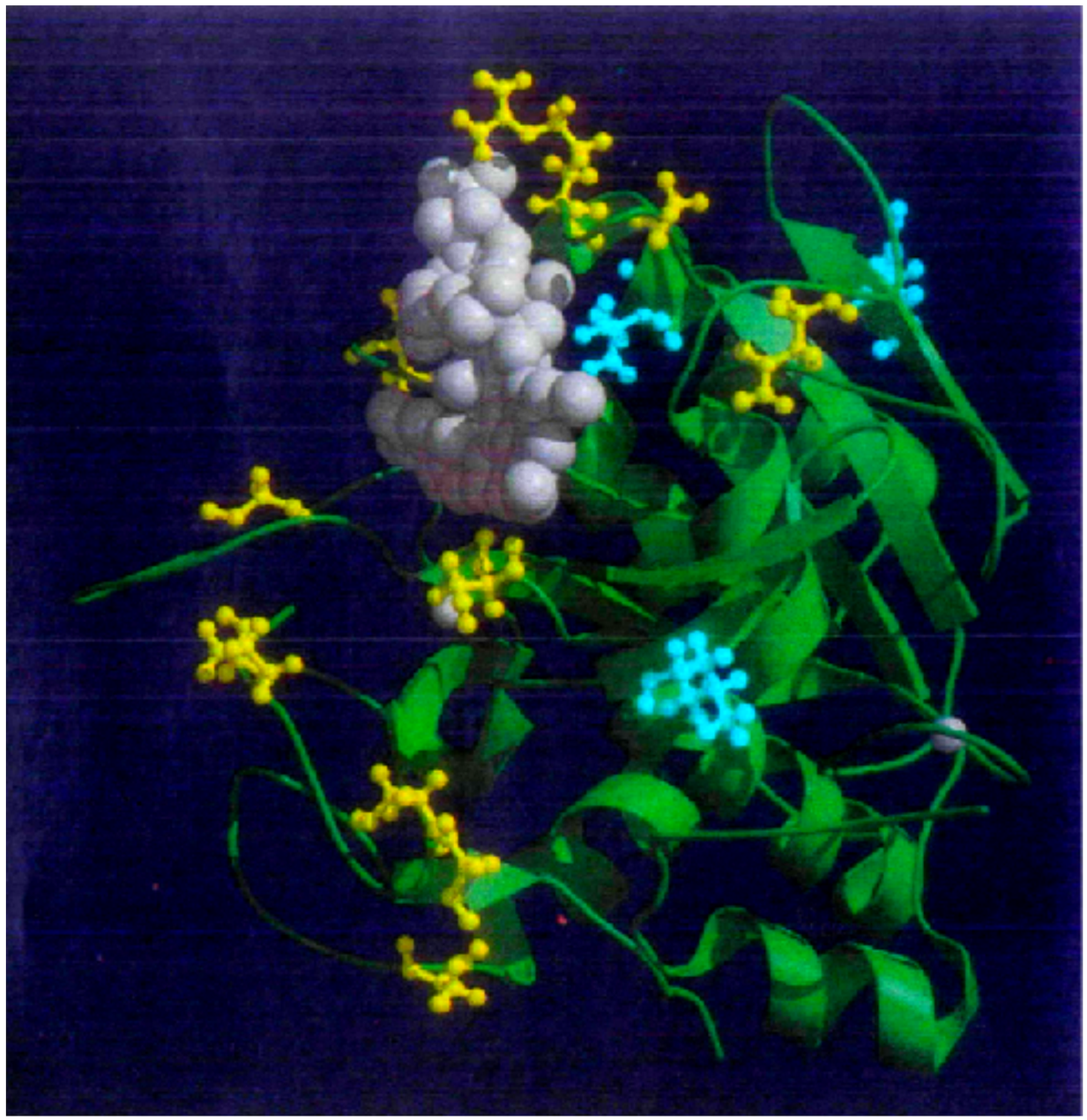
Table I. Total activities of supernatants of *B.subtilis* expressing wild type and variant subtilisins E

Subtilisin E	0% DMF (units/ml)	20% DMF (units/ml)
WT	4.5	0.2
10M	0.27	0.21
12M	1.4	1.55
13M	2.7	3.4

Assay is for hydrolysis of s-AAPF-pNA in 0.1 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0 with and without 20% DMF (v/v).

(10M is what the last paper developed)





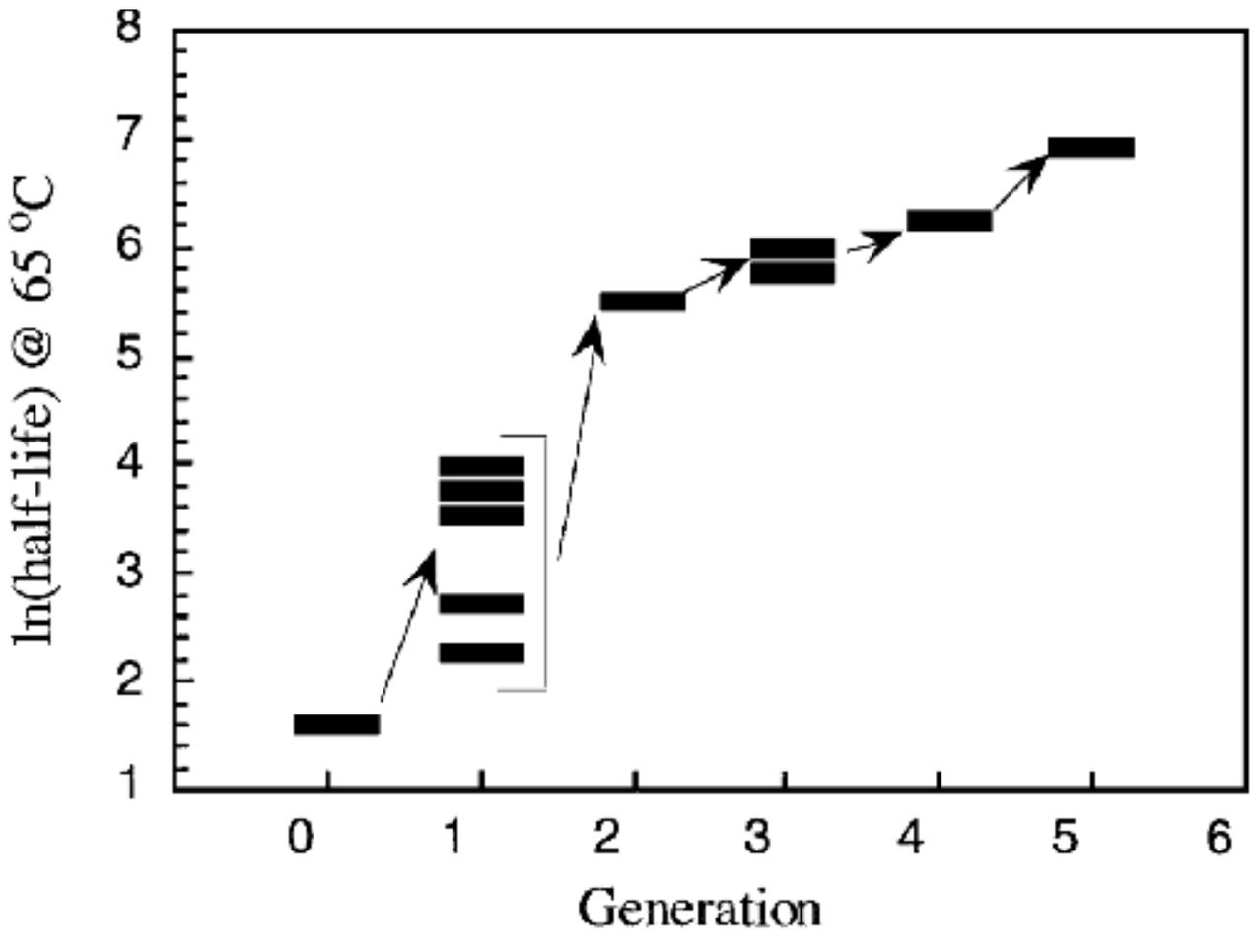
Arnold: Directed Evolution

Protein Engineering vol.12 no.1 pp.47–53, 1999

Directed evolution converts subtilisin E into a functional equivalent of thermitase

Huimin Zhao[1] and Frances H.Arnold[2]

selecting for higher temperature activity



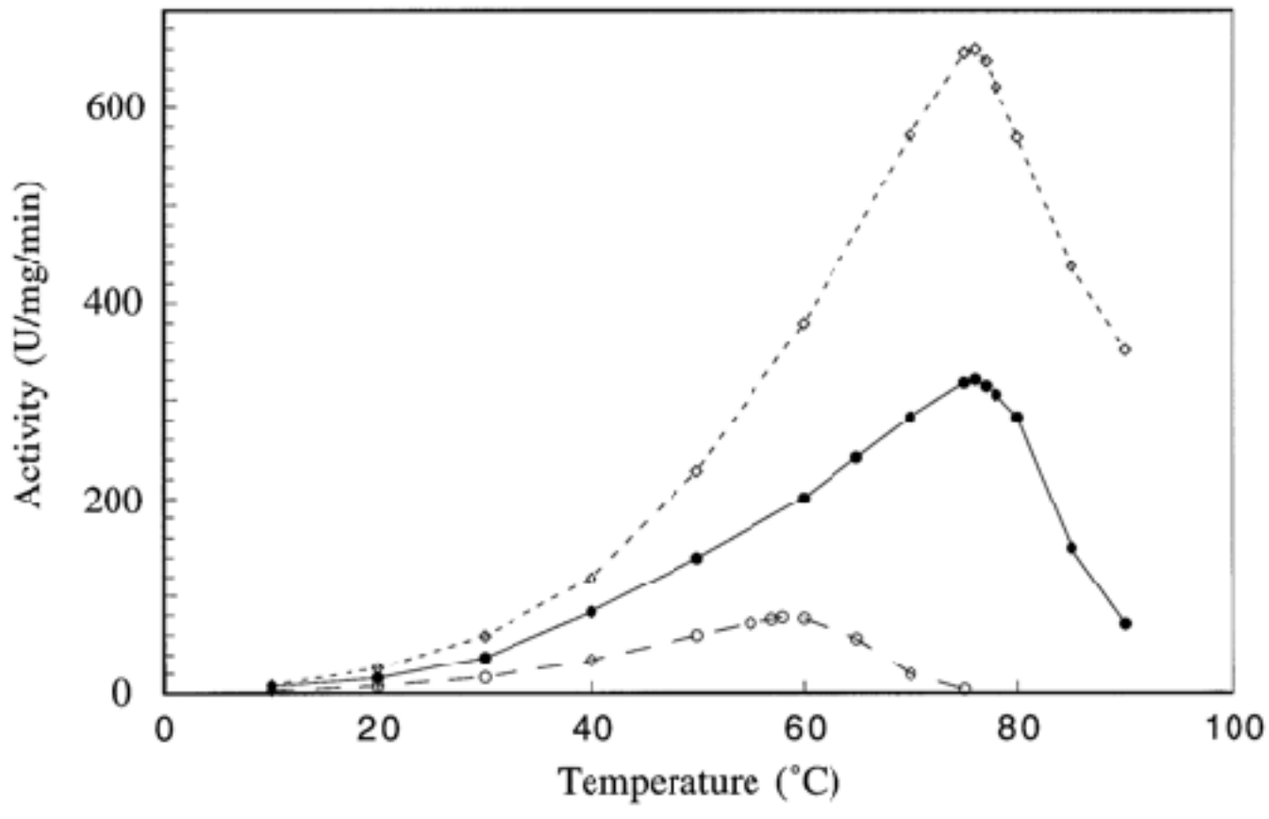


Fig. 3. Activity–temperature profiles of wild-type subtilisin E (– – –), 5-3H5 (—) and thermitase (- - -).

Thermitase differs from subtilisin E at 157 amino acid positions.
However, only eight amino acid substitutions were sufficient to convert subtilisin E into an enzyme equally thermostable.

Arnold: Directed Evolution

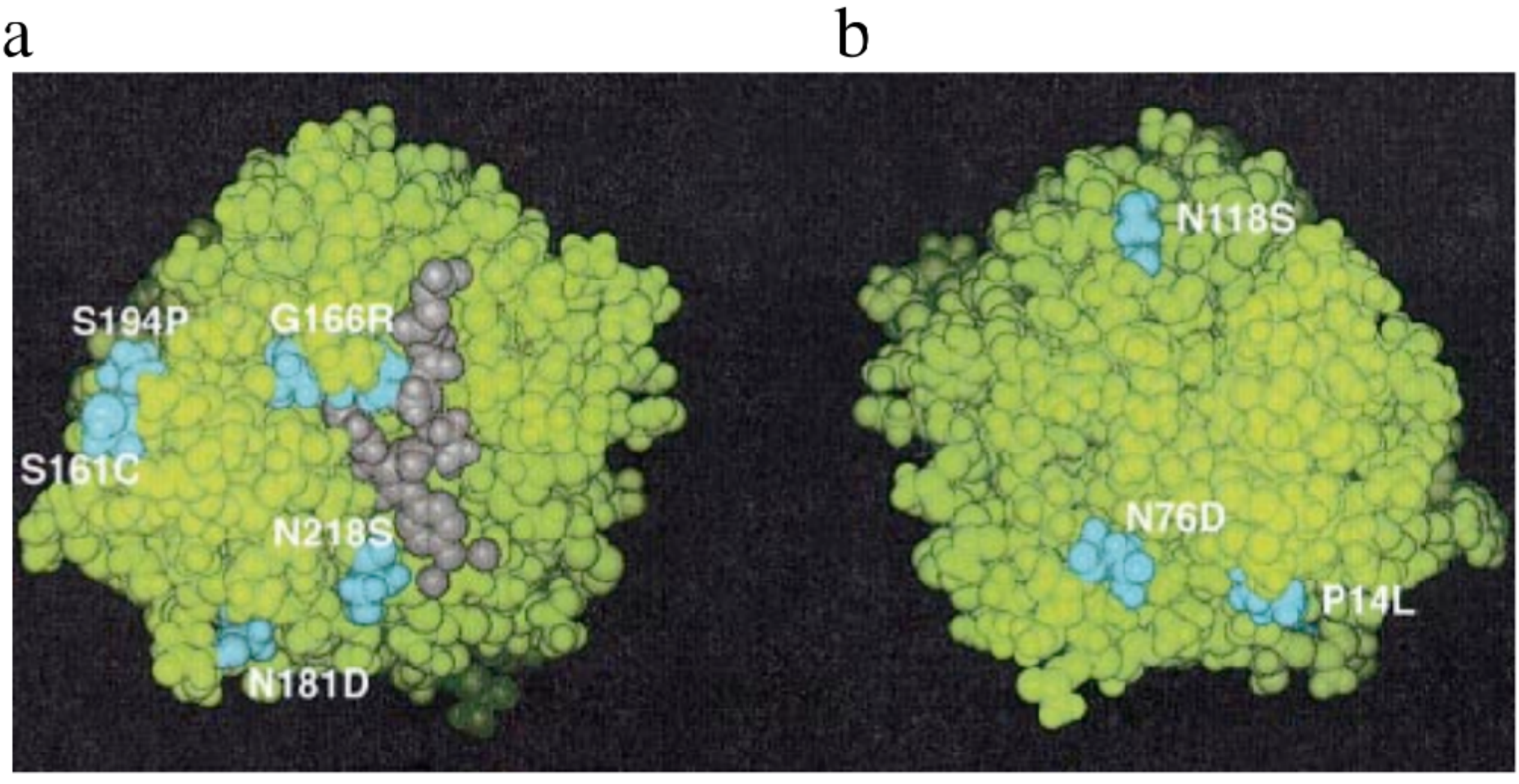
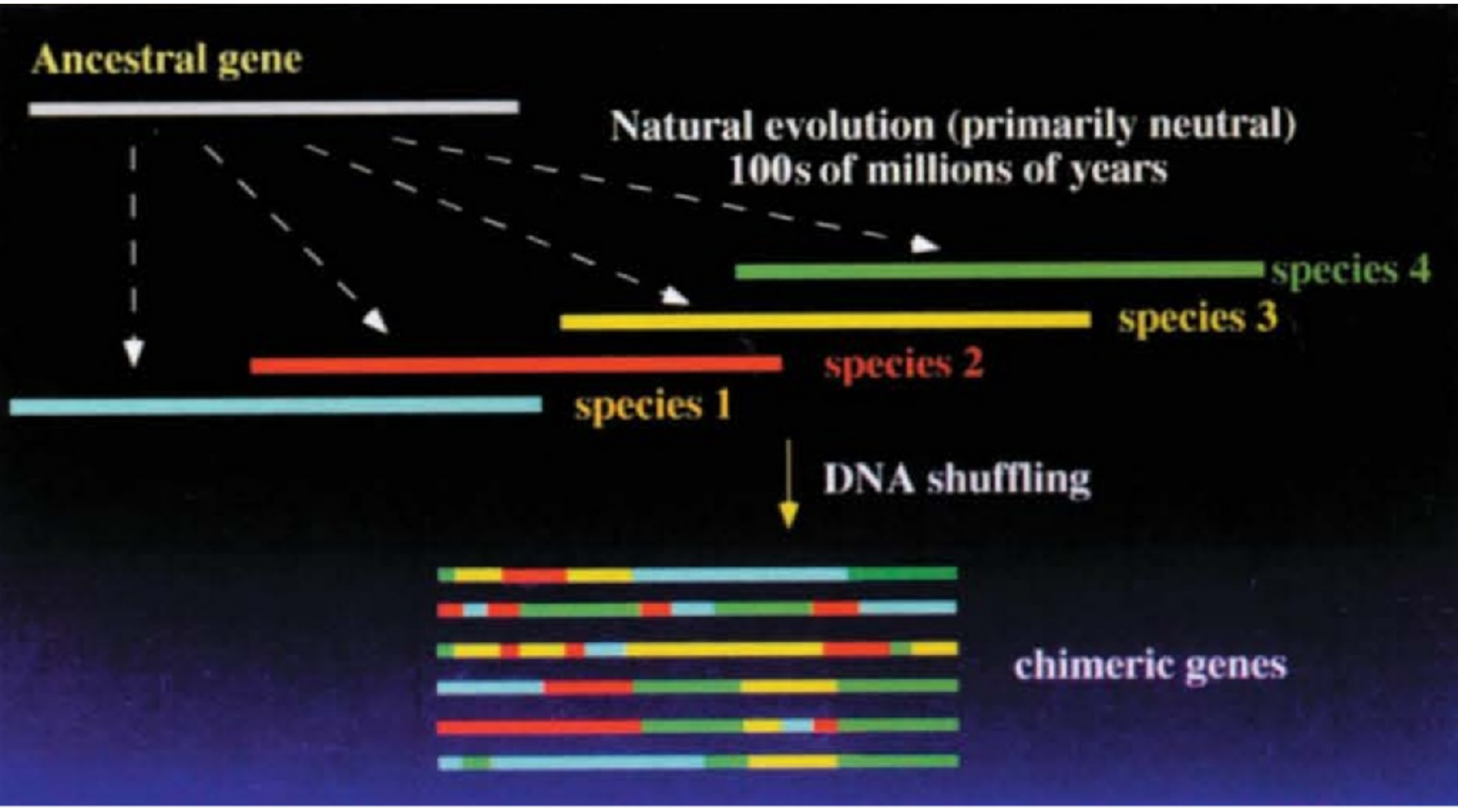


Fig. 6. Space-filling model of 5-3H5 subtilisin E showing the eight thermostabilizing mutations (cyan) and peptide substrate s-AAPF-pNa (gray). (b) View after rotation of (a) by 180°.

Arnold: Directed Evolution

Homolog Shuffling aka Molecular Breeding
invented by Stemmer 1994

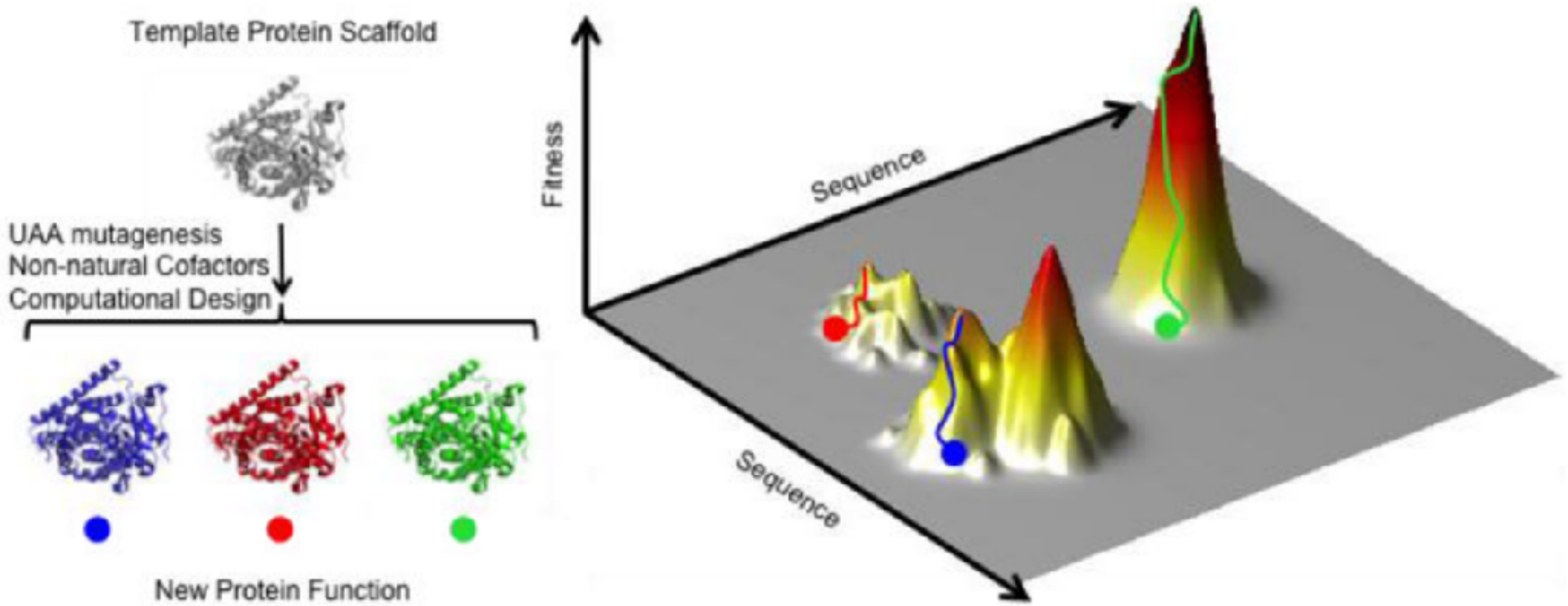
When blind is better:
Protein design by evolution



Arnold: Directed Evolution

Optimizing Non-natural Protein Function with Directed Evolution

Eric M Brustad[1] and Frances H Arnold[1,2]



Arnold: Directed Evolution

Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation

Hyun Joo, Zhanglin Lin & Frances H. Arnold

Cytochrome P450

-superfamily of enzymes containing heme as a cofactor that mostly, but not exclusively, function as monooxygenases
-In mammals, these proteins oxidize steroids, fatty acids, and xenobiotics, and are important for the clearance of various compounds, as well as for hormone synthesis and breakdown
-In plants, these proteins are important for the biosynthesis of defensive compounds, fatty acids, and hormones

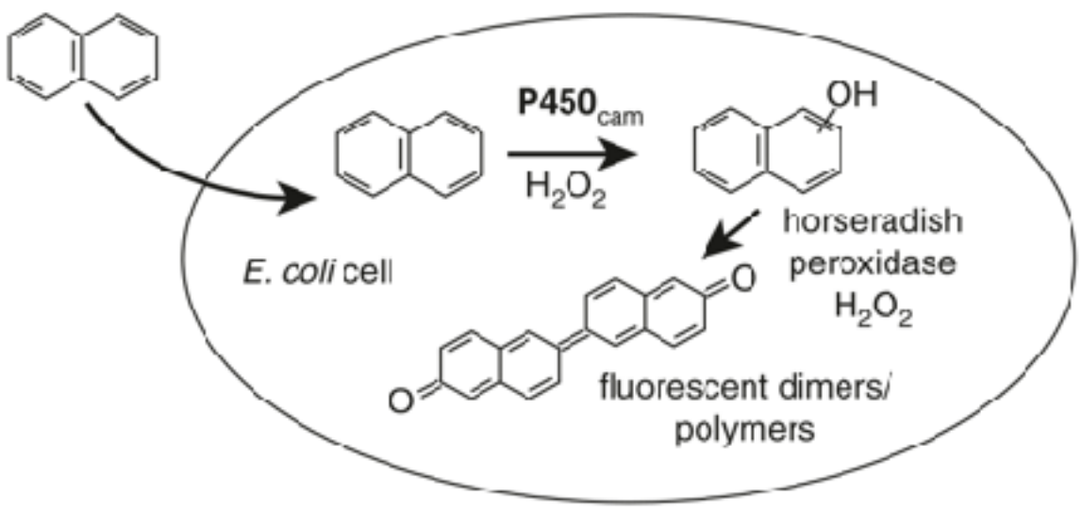


Figure 1 Reaction scheme for detection of active $P450_{cam}$ variants using HRP to generate fluorescent products. The aromatic substrate (here, naphthalene) is taken up by the cells, where it is hydroxylated by the oxygenase. The products of this reaction are oxidatively coupled by HRP, also expressed in the *E. coli*. The product of the coupling reaction is highly fluorescent, and emits at a longer wavelength, relative to the naphthol.

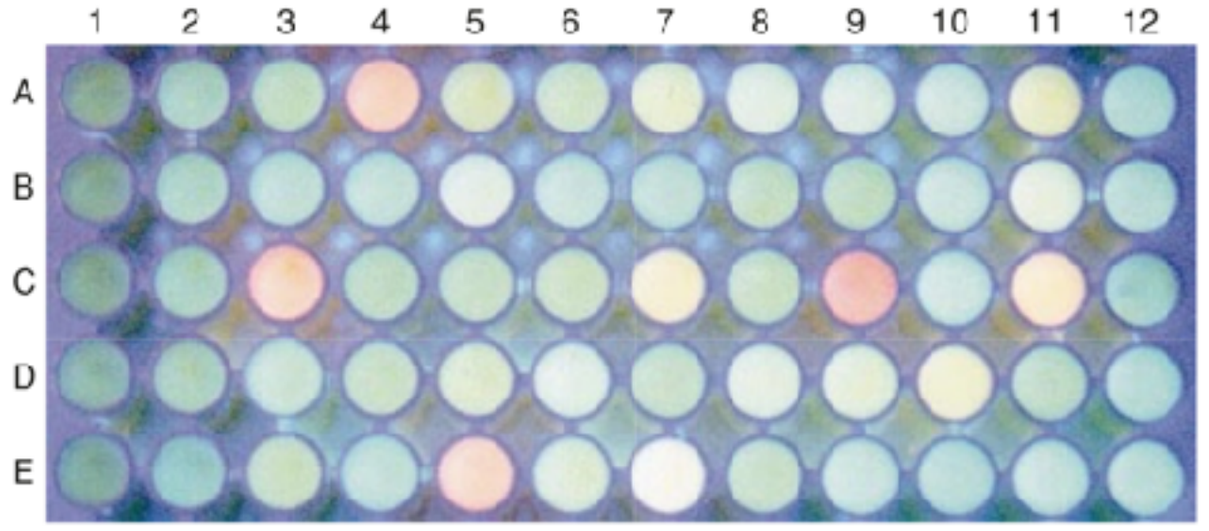


Figure 4 Different colours generated by bacteria expressing HRP and selected $P450_{cam}$ mutants indicating changes in regiospecificity of naphthalene hydroxylation. The first column on the left side (rows A–E) contains control strain *E. coli* BL21 (DE3). The second column contains cells expressing wild-type $P450_{cam}$. The remaining 50 wells contain different $P450_{cam}$ variants selected by fluorescence image scanning.

Arnold: Directed Evolution

Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase

Anton Glieder[1*], Edgardo T. Farinas[2*], and Frances H. Arnold[2†]

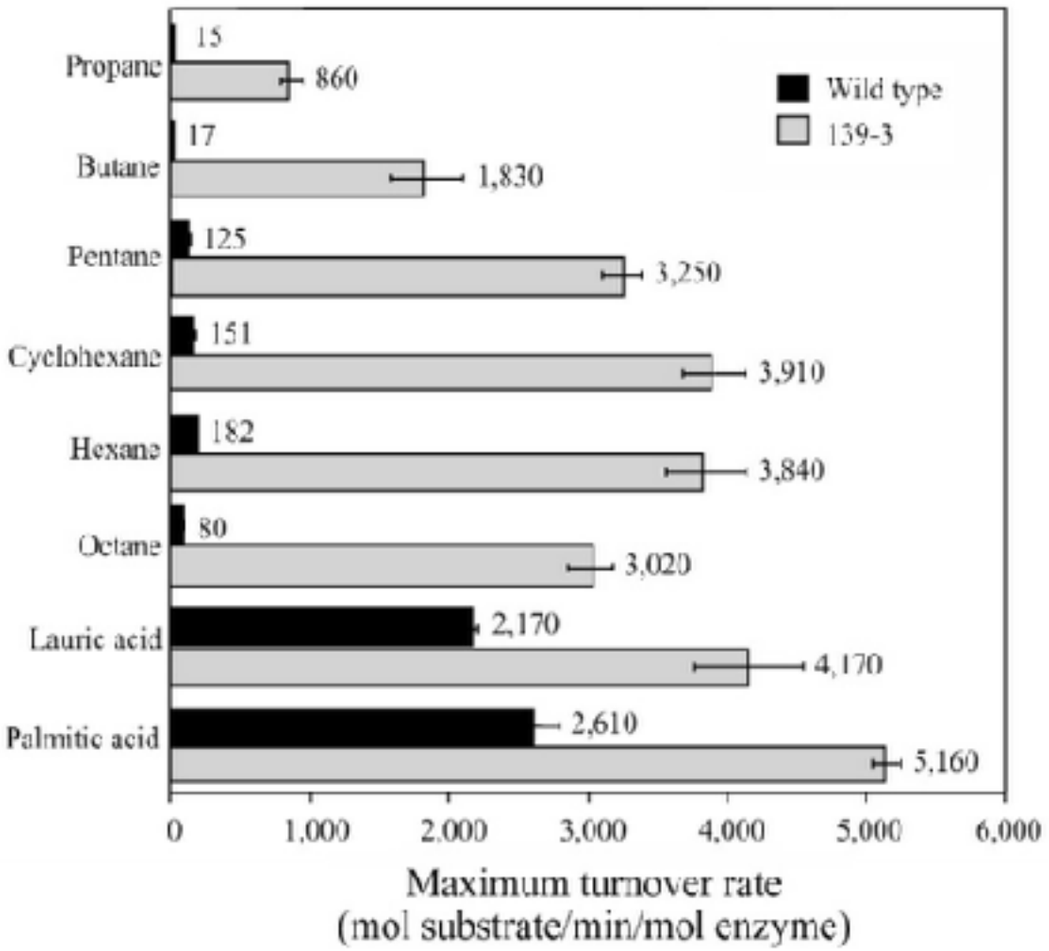


Figure 1. Maximum turnover rates (mol substrate/min/mol enzyme) for P450 BM-3 wild type and 139-3. Substrate concentrations for maximum initial rates of 139-3 (shaded bars) and wild type (black bars) are as follows: propane (saturated solution), butane (saturated solution), pentane (2.5 and 2.5 mM, respectively), hexane (2.5 and 2.5 mM), cyclohexane (2.5 and 2.5 mM), octane (2.5 and 5.0 mM), lauric acid (0.5 and 1.0 mM), palmitic acid (0.5 and 0.5 mM).

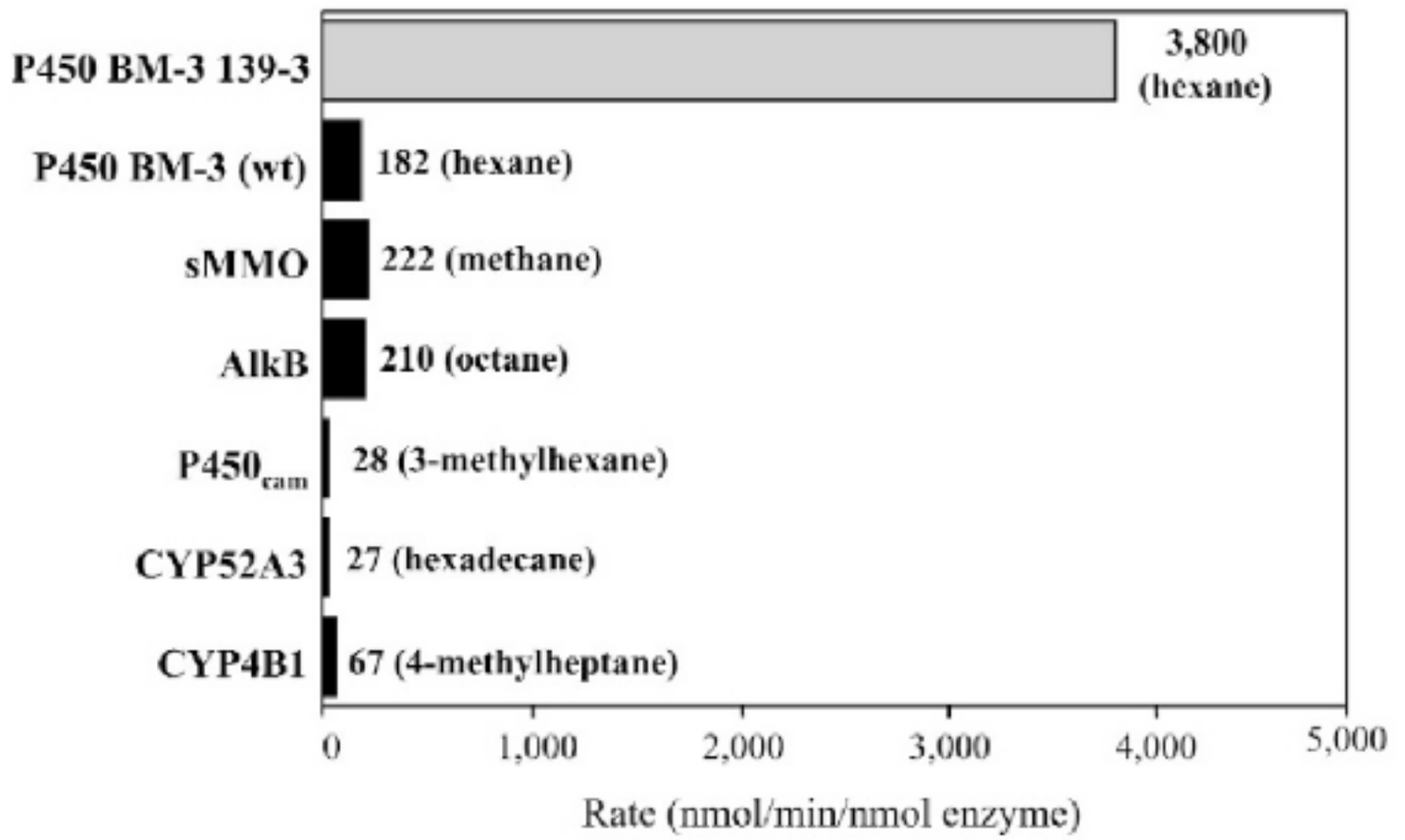


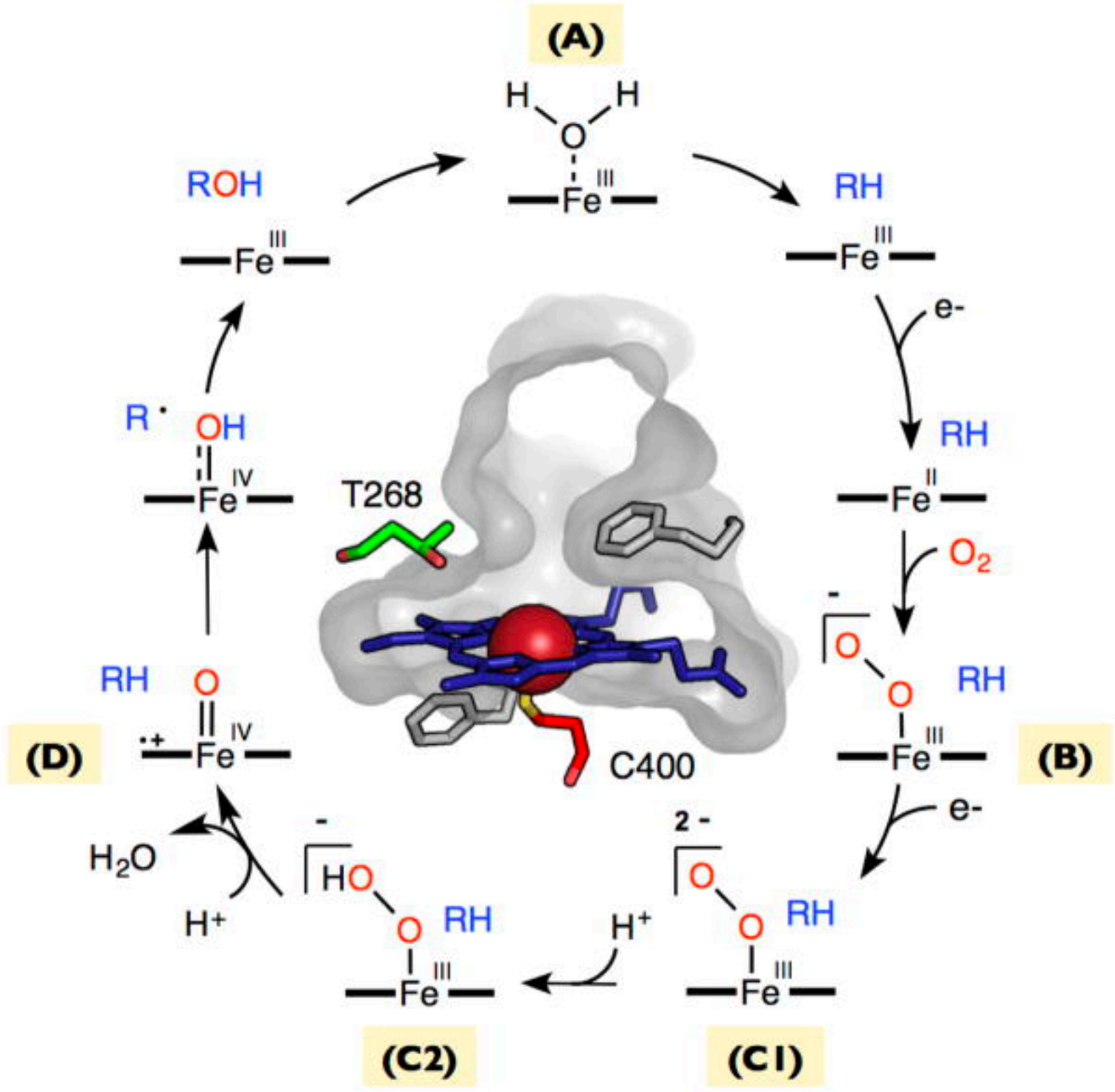
Figure 2. Maximum rates reported for alkane hydroxylation by alkane monooxygenases. Rates for CYP4B1 (ref. 10), CYP52A3 (ref. 7), $P450_{cam}$ (ref. 8), AlkB (ref. 20), and sMMO (ref. 9) were obtained from the literature. Rates for P450 BM-3 wild type and mutant 139-3 were determined in this work.

Arnold: Directed Evolution

Expanding P450 catalytic reaction space through evolution and engineering

John A McIntosh, Christopher C Farwell, Frances H Arnold

Current Opinion in Chemical Biology
Volume 19, April 2014, Pages 126-134



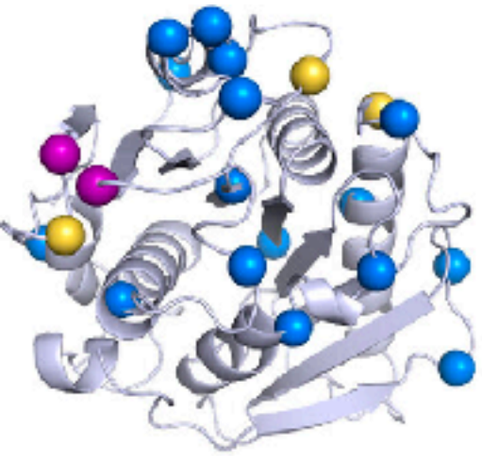
Arnold: Directed Evolution

- Instead of producing pharmaceuticals, plastics and other chemicals using traditional chemistry, which often requires strong solvents, heavy metals and corrosive acids, her idea was to use the chemical tools of life: enzymes. They catalyse the chemical reactions that occur in the Earth's organisms and, if she learned to design new enzymes, she could fundamentally change chemistry.
- Her research group has developed enzymes that transform simple sugars to isobutanol, an energy-rich substance that can be used for the production of both biofuels and greener plastics.
- Arnold and co-workers changed the activity of cytochrome P450 to catalyse a set of reactions for which no specific enzyme was previously available, for example, cyclopropanation. (Cytochrome $P450_{BM3}$)

Arnold: Directed Evolution

- Arnold and co-workers started from a cytochrome P411 variant that performs azide reduction about 100 times more efficiently than nitrene transfer to sulphide. Using directed evolution they produced an enzyme variant that instead efficiently promotes the desired nitrene transfer process.
- Arnold and co-workers evolved a multi-enzyme pathway for carotenoid production in *E. coli*.
- Her lab also showed how whole-cell biocatalysts can be developed for the production of valuable chemicals by using directed evolution to enable the production of L-methionine in *E. coli*.
- Directed evolution of enzymes has become a highly efficient protocol for development of biocatalysts with high specificity, limited side reactions and tolerance of diverse reaction conditions.

Directed Evolution Applications



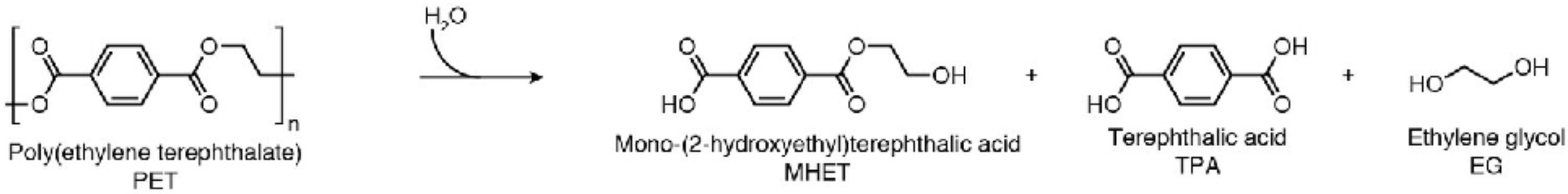
Engineered enzymes

CO_2 capture & utilization

Bioremediation of toxic pollutants

Deconstruction of plastics

Renewable feedstocks



Poly(ethylene terephthalate) PET

Mono-(2-hydroxyethyl)terephthalic acid MHET

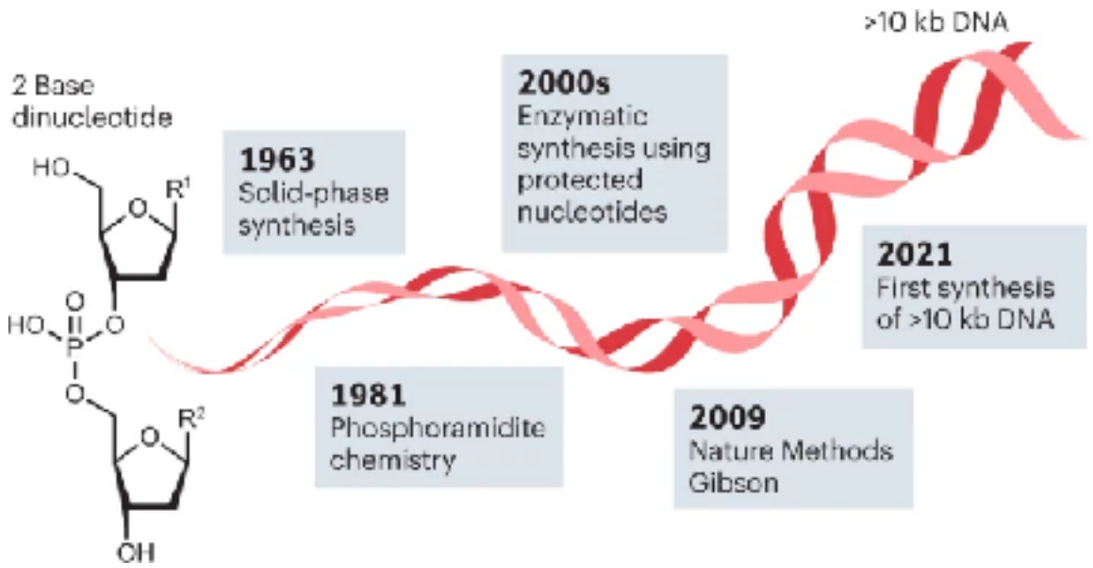
Terephthalic acid TPA

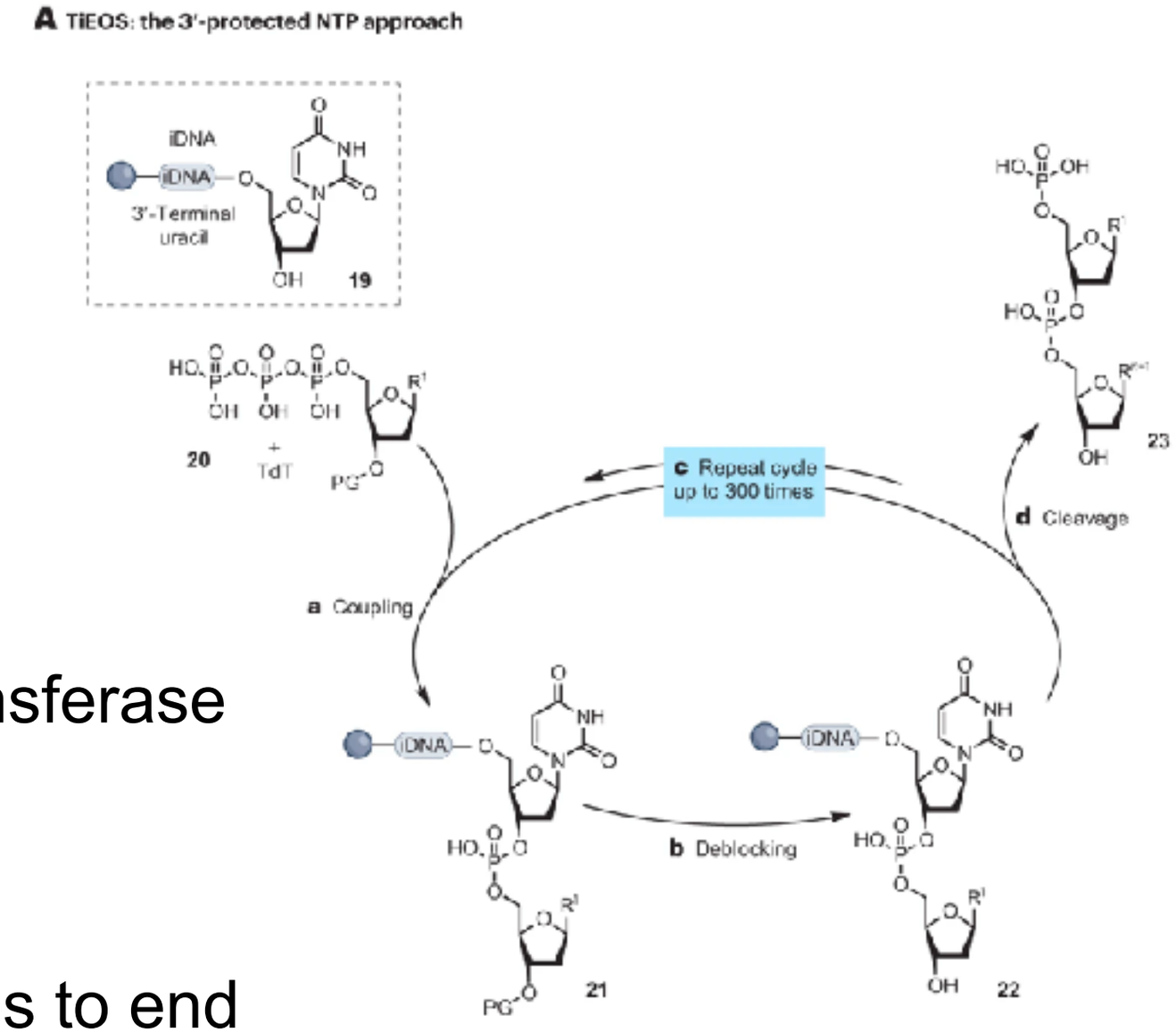
Ethylene glycol EG

Radley, E., Davidson, J., Foster, J., Obexer, R., Bell, E. L., & Green, A. P. (2023). Engineering enzymes for environmental sustainability. *Angewandte Chemie International Edition*, 62(52), e202309305.

Directed Evolution Applications

de novo synthesis of specific DNA oligoes





TdT : terminal deoxynucleotidyl transferase

- DNA polymerase that is template independent
- in other words can add nucleotides to end

Hoose, A., Vellacott, R., Storch, M., Freemont, P. S., & Ryadnov, M. G. (2023). DNA synthesis technologies to close the gene writing gap. *Nature Reviews Chemistry*, *7*(3), 144-161.

Hero of Evolution 2024

Frances Arnold at Caltech in 2021 by Christopher Michel

By Cmichel67 - Own work, CC BY-SA 4.0,

https://commons.wikimedia.org/w/index.php?curid=125897099