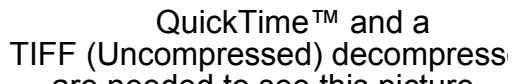
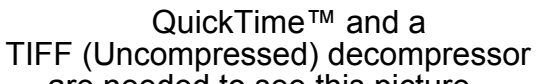
Biocatalysis





RNA Ligase

Trypsin

Aminotransferase

Amanda Garner

September 14, 2007

All enzyme structures from: http://www.ebi.ac.uk/thornton-srv/database/enzymes/

Enzymes

Responsible for the chemistry of life by catalyzing chemical transformations that make/break covalent bonds in cell

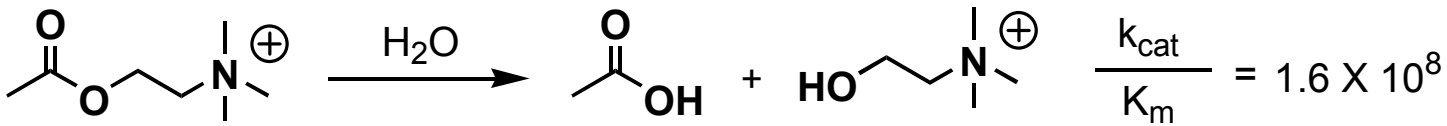
QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Enzyme Specificity and Efficiency

Enzymes exhibit remarkable specificity for substrate and large rate accelerations

- Chiral environment due to side chains (selectivity and specificity)
- Rate constant ~10^6-10^8 $M^{-1} \bullet s^{-1}$
- k_{cat}/k_{uncat} ~ 10^6-10^{12} (up to 10^{17}!)
- Example: Acetylcholinesterase

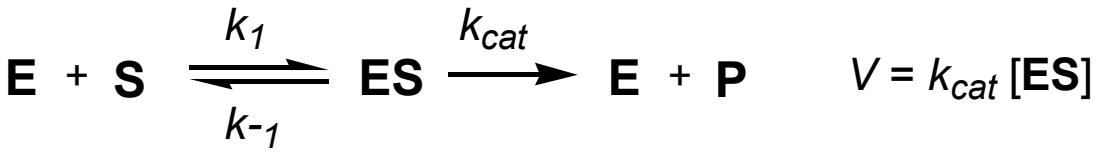
QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.



Acetylcholine Choline

Acetylcholinesterase

Enzyme Kinetics



$V = k_{cat}$ [**ES**]

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

[**ES**] ~ constant so assume steady state: rate of **ES** breakdown = rate of **ES** formation

$$k_{-1}\,[\mathbf{ES}] + k_{cat}\,[\mathbf{ES}] = k_1\,[\mathbf{E}][\mathbf{S}]$$

Several manipulations:

$$V = \frac{k_{cat}\,[\mathbf{E_o}][\mathbf{S}]}{K_m + [\mathbf{S}]} = \frac{V_{max}\,[\mathbf{S}]}{K_m + [\mathbf{S}]}$$

Michaelis-Menten equation

K_m = substrate affinity for enzyme ([**S**] at $V = 0.5\ V_{max}$);
(low K_m = tight binding; high K_m = weak binding)
k_{cat} = turnover number

Enzyme Efficiency

Fischer (1890) - “Lock and Key” model: binding of **S** results in its activation to effect catalysis

Pauling (1948) - Transition State model:

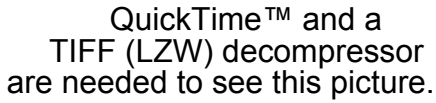
Lowering of E_a because has higher affinity for TS than GS

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Other factors:

1. Increased local [**S**] to hold atoms in correct orientation
2. Capability for simultaneous acid and base catalysis
3. Capability for covalent catalysis (ex. Histidine residue)

Industrial Use of Enzymes: Biocatalysis



TRENDS in Biotechnology **2007**, *25*, 66-71

Generation of New Enzymes

GOAL - Discover enzymes with unique and enhanced properties

1. Improve enantioselectivity
2. Tune and alter substrate specificity
3. Enhance activity
4. Discover new activities
5. Improve stability (ex. temperature, structure)
6. Ability to function in organic solvent

Arnold, F. A. *Curr. Opn. Chem. Biol.* **1999**, *3*, 54-59.

Rational Design

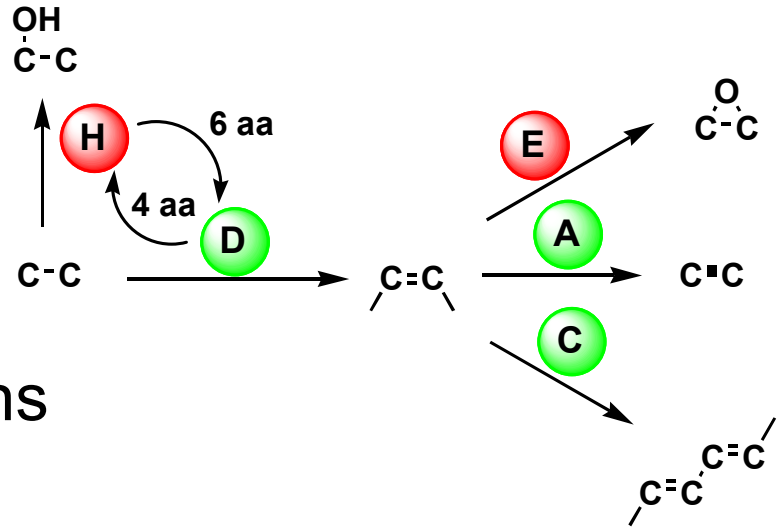
In Nature, new enzymes evolved through minor modifications of the active site

Method: Rational Design (fine tune existing enzymes through site-directed mutagenesis)

Example: di-iron enzyme family

7 aa differ b/t H and D;

can convert function through mutagenesis at 4/6 aa positions



Drawback: difficult to discern aa responsible for substrate preference, stability, activity

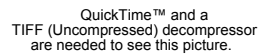
Shaklin, J. *Science* **1998**, *282*, 1315-1317; Arnold, F. A. *Nature* **2001**, *409*, 253-257.

Evolution

Natural selection or *survival of the fittest* (Darwin, 1859):

process by which favorable mutations are selected for over time

In Nature, this is an uncontrolled process over millions of years

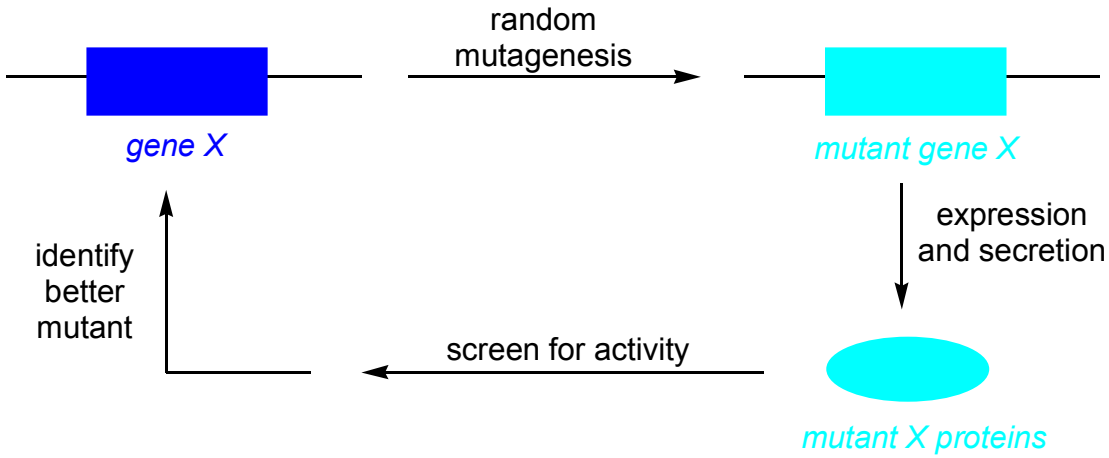


Can evolutionary processes be directed towards a certain defined goal in an efficient manner (days/weeks)?

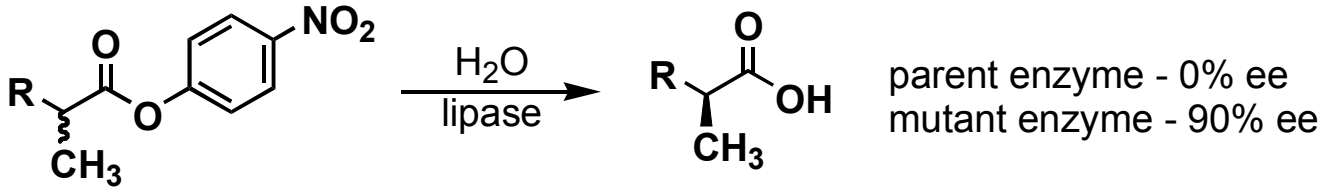
Directed Evolution = Irrational Design

"Breeding" of enzyme for certain properties

Involves iterative process of making random mutations (e.g. site mutagenesis, error-prone PCR) and screening the pool of mutants for the desired property



Example:



parent enzyme - 0% ee
mutant enzyme - 90% ee

Reetz, M. T.; Jaeger, K-E. *Chem. Biol.* **2000**, *7*, 709-718.

DNA Shuffling

Nature uses both mutation and recombination to increase number of combinations of genes

DNA shuffling: homologous recombination of pools of selected mutant genes by random fragmentation and PCR reassembly (greatly increase # of mutations)

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Tobin, M. B. *Curr. Opn. Struct. Biol.* **2000**, *10*, 421-427.
Stemmer, W. P. C. *Nature*, **1994**, *370*, 389--391; *PNAS*, **1994**, *91*, 10747-10751.

DNA Shuffling of Chimaeric Genes

Shuffling of gene family to accelerate evolution

Example: family of class C cephalosporinases

Test for moxalactam resistance by DNA shuffling

Single gene shuffling - 8-fold ↑ in resistance

Multi-gene shuffling - 270-540-fold ↑ in resistance

(best mutant contained segments from 3/4 genes shuffled)

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Stemmer, W. P. C. *Nature*, **1998**, *391*, 288-291.

Directed Evolution Toward New Catalytic Activity

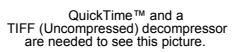
QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

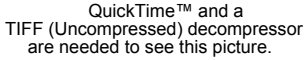
Change catalytic activity of existing protein scaffold glyoxalase (metallohydrolase) to β-lactamase with $k_{cat}/K_m = 1.8 \times 10^2\ M^{-1} \cdot s^{-1}$

Use technique combining directed evolution with insertion, deletion, and substitution of gene segments

Kim, H-S. *Science* **2006**, *311*, 535-538.

Catalytic Antibodies





Jencks (1967): antibody raised against analog of TS of reaction to catalyze reaction by $\downarrow E_a$ by recognizing and binding to transient TS structure

First example in 1986 independently by Schultz and Lerner:

Raise hapten (antigen) mimicking TS against antibody to screen for catalytic activity

>100 reactions accelerated incl. new and disfavored reactions

Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1967.
Schultz, P. G. *Science*, **1986**, *234*, 1570.; Lerner, R. A. *Science*, **1986**, *234*, 1566.

Catalytic Antibodies - Transition State Analogs

Hapten mimics bond orders, lengths, angles, expanded valences, charge distribution, geometry,etc. of transition state

Example:

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Phosphonamidate mimics tetrahedral intermediate

Lerner, R. A. *Science*, **1988**, *241*, 1188.
Janda, K. D. *Bioorg. Med. Chem.* **2004**, *12*, 5247-5268.

Catalytic Antibodies - "Bait-and-Switch"

Place point charge (bait) in close proximity to or in direct substitution for functional group expected to transform substrate (switch)

Example:

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

← Bait

→ Switch

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274. Janda, K. D. *PNAS*, **1998**, *95*, 5971.

Catalytic Antibodies - Reactive Immunization

Highly reactive hapten that undergoes reaction in antibody-combining site during immunization

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Lerner, R. A.; Janda, K. D. *Science*, **1995**, *270*, 1775.

Enzymes vs. Catalytic Antibodies

Enzymes:

- Rate constant ~10^6-10^8 L/mol•s
- k_{cat}/k_{uncat} ~ 10^6-10^{12}

Catalytic Antibodies:

- Rate constant ~10^2-10^4 L/mol•s
- k_{cat}/k_{uncat} ~ 10^3-10^5

Best antibody = poor enzyme; why?

- Antibody raised for tight binding not catalytic efficiency (may tightly bind pdt if structurally similar)
- Hapten binding site solvent accessible
- Smaller time scale of evolution (weeks to months)
- Immunoglobulin fold not common structure in enzymes

Hilvert, D. *Annu. Rev. Biochem.* **2000**, *69*, 751-793. Janda, K. D. *Bioorg. Med. Chem.* **2004**, *12*, 5247-5268.

Szostak Method: mRNA Display

Main barrier to protein evolution is difficulty of recovering information encoding protein sequence after protein is translated

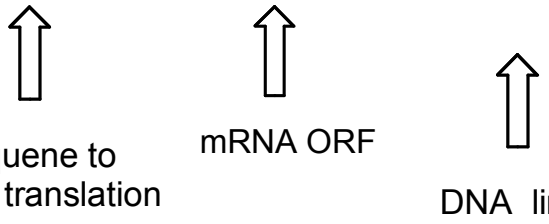
mRNA Display: mRNA directly attached to protein it encodes via stable covalent linkage

Allows for generation of large complex libraries (>10^{12}) *in vitro*

Example:

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

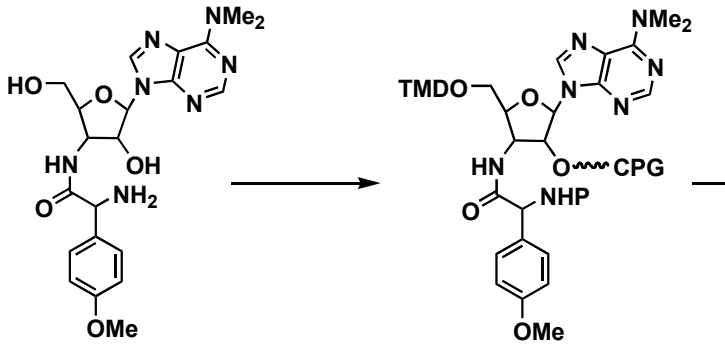
QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.



P = puromycin to stop translation

Szostak, J. W. *PNAS*, **1997**, *94*, 12297-12302.

mRNA Display



QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

RNA synthesis;
ligation

P = puromycin; antibiotic that mimics aminoacyl end of tRNA to inhibit translation

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Szostak, J. W. *PNAS*, **1997**, *94*, 12297-12302.

mRNA Display

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

1. Ribosome initiates translation on mRNA and translocates to end of template
2. Ribosome reaches end of ORF and translation stalls at RNA/DNA junction (no stop codon)
3. Linker loops around ribosome and P enters A site to attach to newly formed protein
4. Incubate with complementary linker sequence attached to biotin and isolate on streptavidin beads

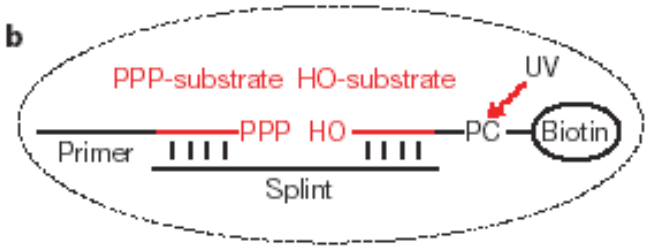
Szostak, J. W. *PNAS*, **1997**, *94*, 12297-12302.

Enzyme from Non-Catalytic Scaffold

Current protein evolution requires extensive knowledge of enzyme's mechanism of activity (not random)

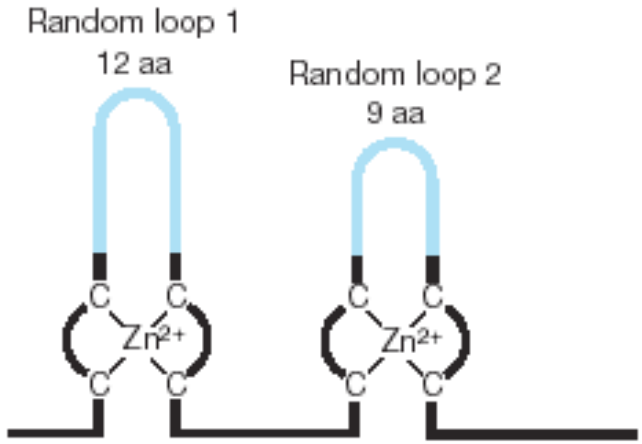
De novo creation of enzymatic active from naïve protein library of very high diversity (>10^{12} unique sequences) using mRNA display

Screen for enzymes catalyzing RNA ligation:



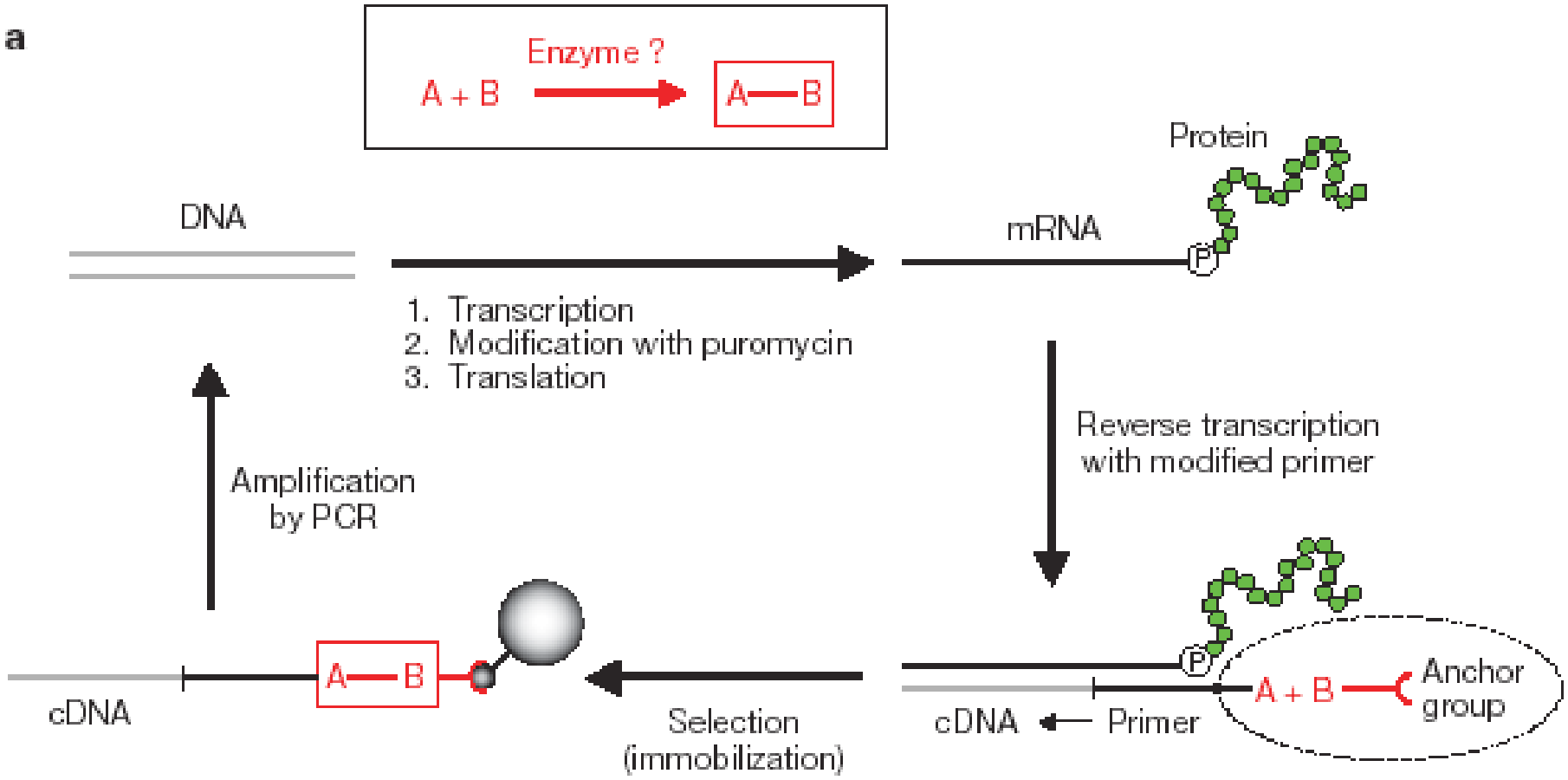
Use 2 loops of retinoid-X-receptor (RXRα)

(prev. used to isolate ATP-binding proteins)



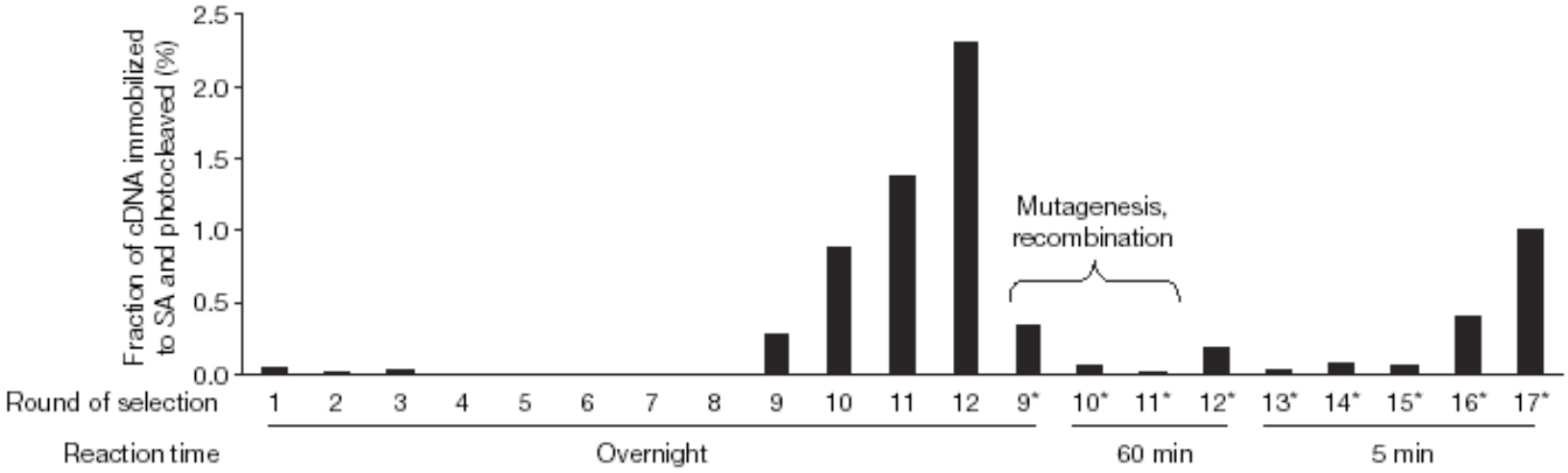
Szostak, J. W. *Chem. Biol.* **2006**,*13*, 139-147. Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold



Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold



1. *In vitro* selection of catalytic activity

   9 rounds - 0.01→0.3% selected

   12 rounds - 2.3% selected

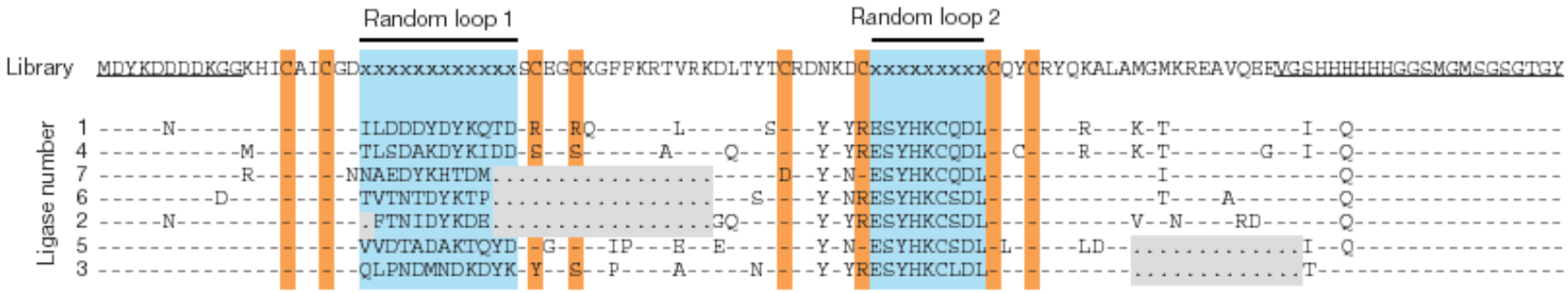
2. Directed evolution

   After round 8, subject to recombination and random mutagenesis + error-prone PCR amplification (9^{*}-17^{*})

   ↑ selection pressure by ↓ time allowed for reaction (select for most efficient enzyme)

Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold



Loop 1 (12 aa): DYKXXD at varying positions in 57% (resembles recognition site for antibody used for purification; may not be important)

Loop 2 (9 aa): 4 aa conserved 100%, 4 aa conserved 86-90%, 1 aa conserved 50%

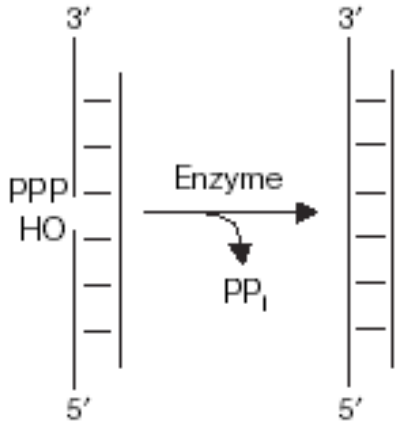
Non-loop region: low conservation of C used to coordinate Zn^{2+} in original structure, 2 large deletions observed (may indicate large structural rearrangement of evolved enzymes)

Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold

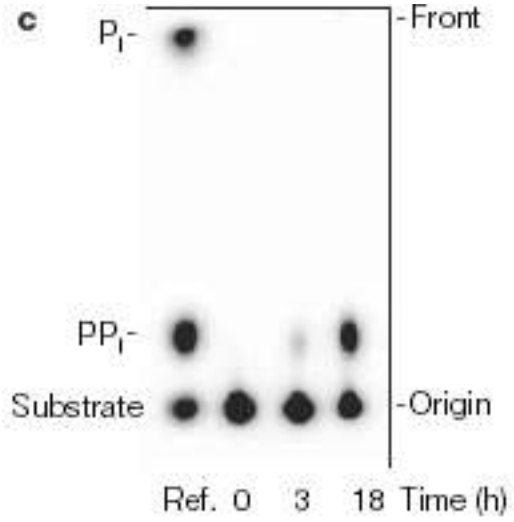
Chose Ligase 4 (most active) to characterize:

Incubate fusion enzyme + PPP-substrate + HO-substrate + splint



1. <u>Test for presence of pyrophosphate:</u>

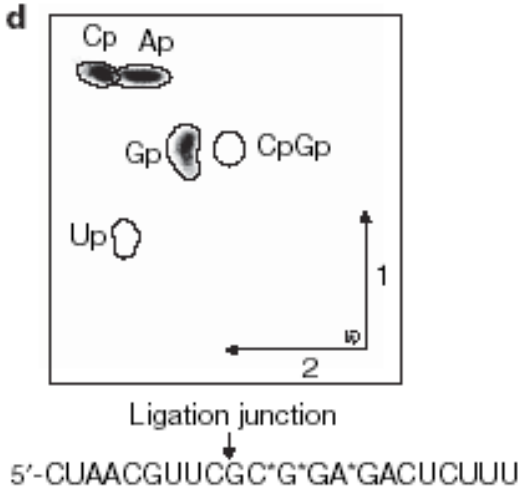
Label with ^{32}P to detect



Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold

2. Test for requirements of the reaction:



Incubation time: Lane 1 = 1h, Lane 2 = 3h, Lane 3 = 10h

No splint: Lane 4

5'-P (not PPP): Lane 5

5'-OH (not PPP): Lane 6

Wild-type RXRα: Lane 7

All nucleobases successful at 3' of HO-substrate

Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold

3. Effect of metals on activity:

   Requires Zn^{2+} and Na^{+}/K^{+} (not only 2+ metals)

   2.6 Zn^{2+}/enzyme (required for structure or catalysis)

4. pH effect:

   Strong pH dependence with optimum pH = 7.6 (could rely on acid/base catalysis from aa residues)

Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold

5. Rate acceleration:

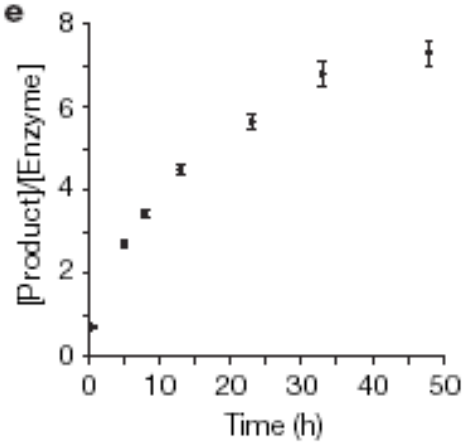
Uncatalyzed reaction only takes place with Mg^{2+} but catalyzed faster without Mg^{2+}

Measure *k* in absence of Mg^{2+}:

$k_{obs(noncat)} < 3 \times 10^{-7}\ h^{-1}$

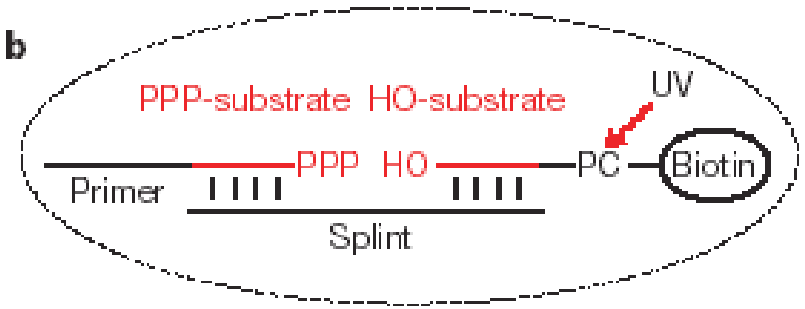
$k_{obs(cat)} = 0.65 \pm 0.11\ h^{-1}$ (2×10^{6}-fold faster!)

Observe multiple turnover:



Szostak, J. W. *Nature*, **2007**, *448*, 828-833.

Enzyme from Non-Catalytic Scaffold



No natural enzyme known to catalyze ligation reaction of 5' PPP-substrate to 3' HO-substrate except ribozyme

First use of mRNA-display to select for new enzyme activity

Isolated enzyme with rate enhancement of 2 million-fold

Szostak, J. W. *Nature*, **2007**, *448*, 828-833.