Enzymes as machines: how they work

Biophysical Society Summer Course

26 June 2014

Charlie Carter

They are Just as Scared of You,
As You are of Them

Adapted from:
Steve Cote, Chapel Hill artist

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Readings

- Nelson, P., *Biological Physics,* Chapter 10
- Fersht, A. *Structure and Mechanism in Protein Science,*
  - Chapter 1(F-G), p. 38-50 (Structures of ES complexes, mobility)
  - Chapter 3, p. 103-129 (Basic Equations of Enzyme Kinetics)
  - Chapter 4, p. 132-167 (Individual Rate Constants)
  - Chapter 7, p. 216-242 (Detection of Intermediates)
  - Chapter 12, p. 349-375 (E-TS Complementarity, Binding Energy)

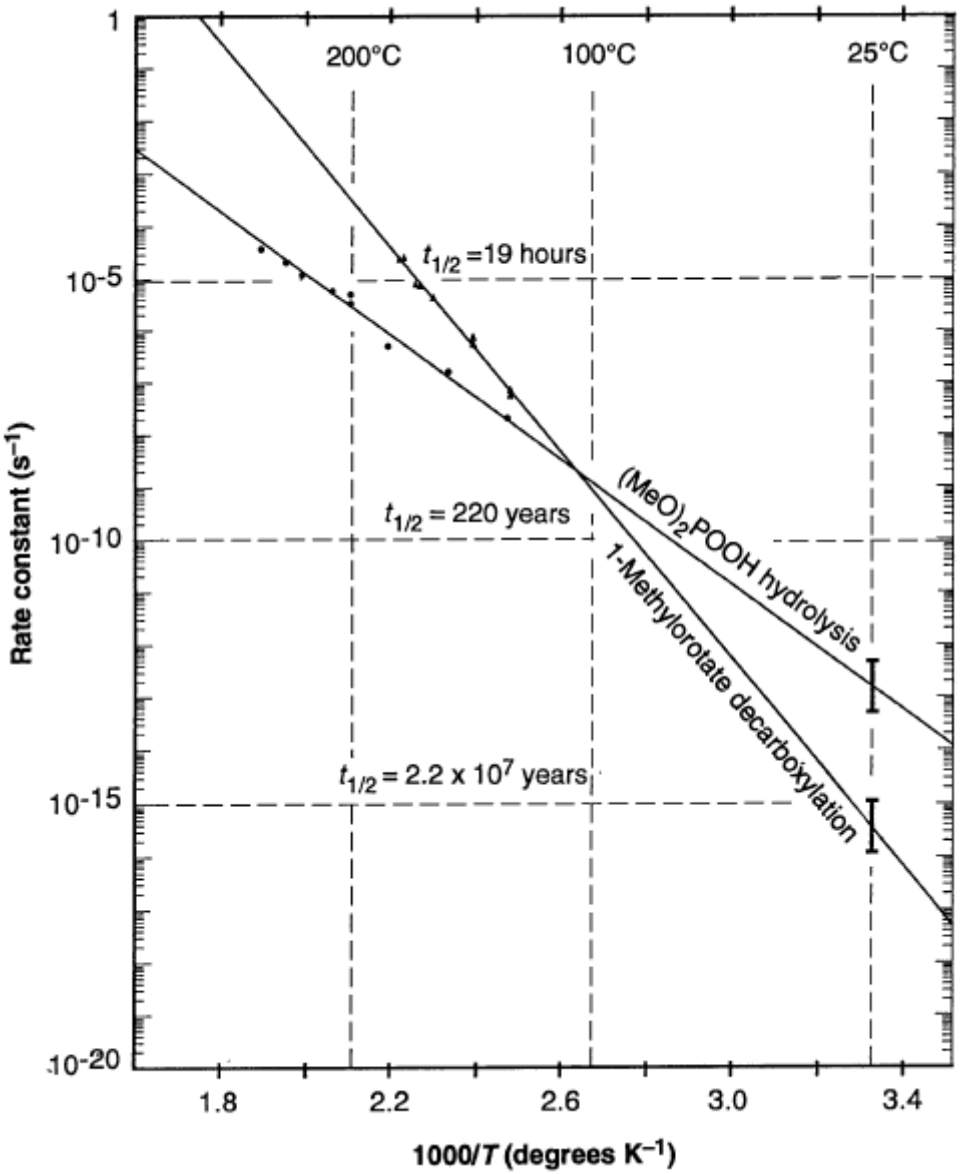
Goals

- Understand why chemistry is slow
- Understand rate acceleration in terms of transition-state theory, equilibrium constants
- Michaelis-Menten steady-state approximation
  - k_{cat}/Km, the apparent 2nd order rate constant; proficiency
  - Specificity for competing substrates (the specificity constant)
- Two structural case-studies: subtilisin and cytidine deaminase
  - Differential binding: an expression of molecular discrimination
  - Strategic use of ΔG_R
  - Stereochemical bases for TS stabilization
  - Transition state analog inhibitors as equilibrium models of catalytic rate enhancement
- Directed mutation, pre-steady state kinetics, and mechanism
- Long-range coupling

Why is *uncatalyzed* chemistry so Slow?

- It's cold! (Rates about double for every 10°C)
- Reactants, products are connected via the ***transition state.***
- Rates $\propto$ [transition states]
- Transition states are very rare for several reasons:
  - Chemistry *exchanges* one bonded state for another
  - Bonded states are *stable stationary points for electrons*
  - Transition states involve *altered charge (electron) distributions*
  - Transition states involve *strained stereochemistry*
- Water *prefers* reactants, products to TSs (most reactions are much faster in the gas phase)!!!

Chemistry is slow because it's *cold!*



Radzicka and Wolfenden, *Science*, **267**:90-93

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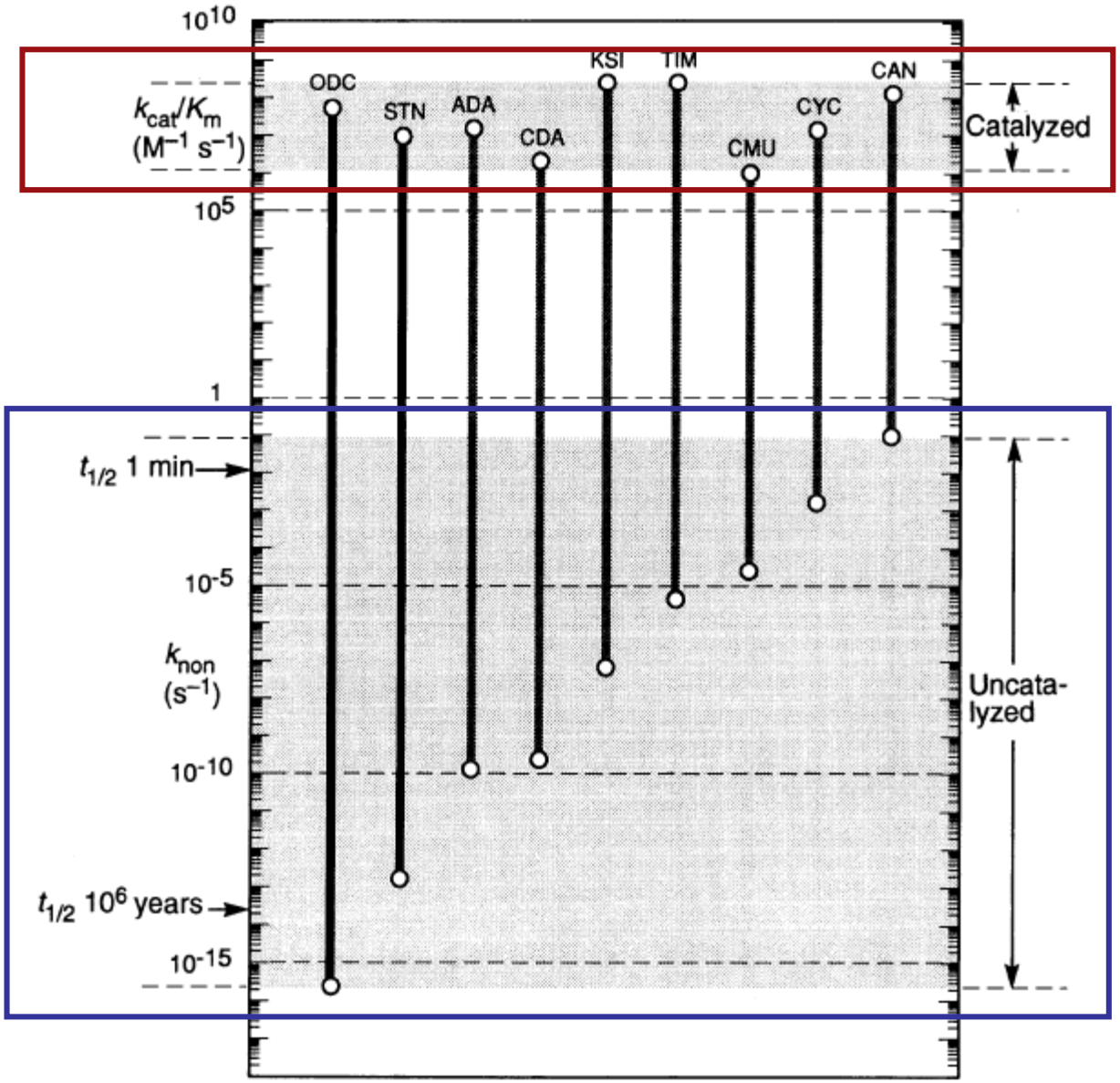
Enzymes are (only) as proficient as they must be!



Catalyzed rates are uniform

Uncatalyzed rates vary widely

Radzicka and Wolfenden, *Science*, **267**:90-93

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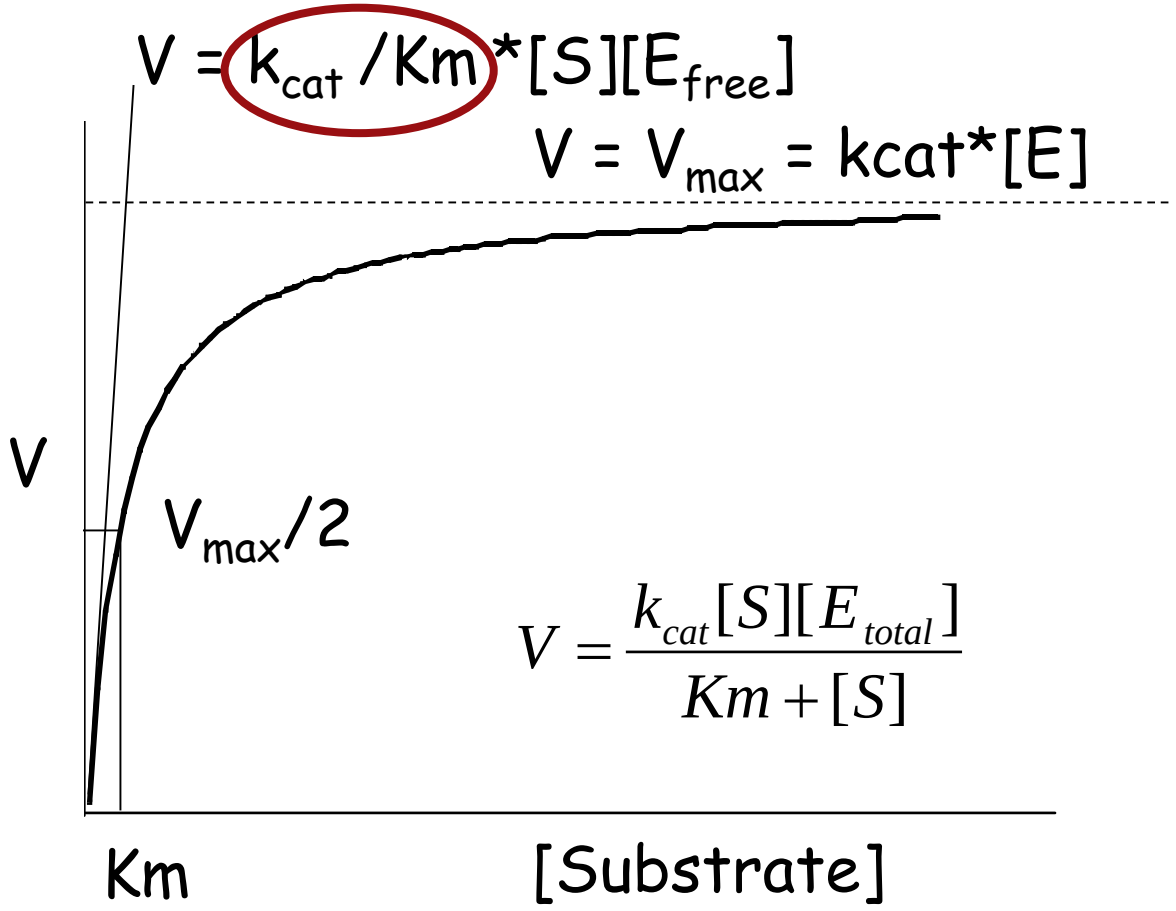
The steady state approximation, saturation, and Michaelis-Menten kinetics



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k_{cat}/Km, the *apparent 2nd order rate constant*

- The ratio of k_{cat} to Km is the most reliable estimate for catalytic proficiency.
- It is the rate constant for the ***second-order*** process $[E_{free}] + [S] = [E_{free}] + [P]$:
  - $rate = k_{cat}/Km * [E_{free}] * [S]$.
- It also provides a quantitative comparison between competing substrates, and hence has been called the "specificity constant".

Chemical reaction rates are governed by $\Delta G^{\ddagger}$

- **Arrhenius**: rate constants should vary with temperature in the same way as equilibrium constants (ie., the van't Hoff law: $\frac{d\ln K}{dt} = \frac{\Delta H}{RT^2}$).

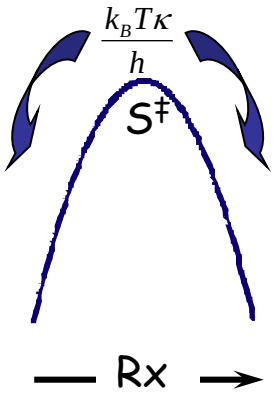
$$\frac{d\ln k}{dt} = \frac{\Delta H^{\ddagger}}{RT^2}; d\ln k = \frac{\Delta E^{\ddagger}}{RT}\frac{dt}{T}; \ln k = \frac{-\Delta E^{\ddagger}}{RT} + \ln A; k = A * e^{\frac{-\Delta E^{\ddagger}}{RT}}$$

  - A is a constant of integration for Arrhenius, who called it a "frequency factor"
- **Eyring**: transition state configuration cannot survive bond-length oscillations. This allows calculation of absolute rates!

$$-\frac{[dA]}{dt} = [Substrate^{\ddagger}] \times Rate\,of\,Crossover \times transmission\,coefficient, \kappa$$

$$Rate\,of\,Crossover = \nu = \frac{E}{h} = \frac{k_B T}{h}, (\,frequency\,of\,bond\,vibration)$$

$$k = \frac{k_B T \kappa}{h} \times \exp\left(\frac{-\Delta \mathbf{G}^{\ddagger}}{RT}\right) = \frac{k_B T \kappa}{h} \times \exp\left(\frac{\Delta \mathbf{S}^{\ddagger}}{R}\right) \times \exp\frac{-\Delta \mathbf{H}^{\ddagger}}{RT}$$



- **Kramers**: "Thermally activated escape from a metastable state is nothing else but the Brownian motion of a fictitious particle along a reaction coordinate leading from an initial to a final locally stable state. In order to overcome the energetic barrier separating the two states, the particle has to "borrow" energy from its surroundings, an extremely rare event if as is usually the case the activation energy is much larger than the thermal energy."
  - (Pollack & Talkner 2005 Chaos, **15)**
  - Catalysis involves *diffusive motion of the enzyme.*

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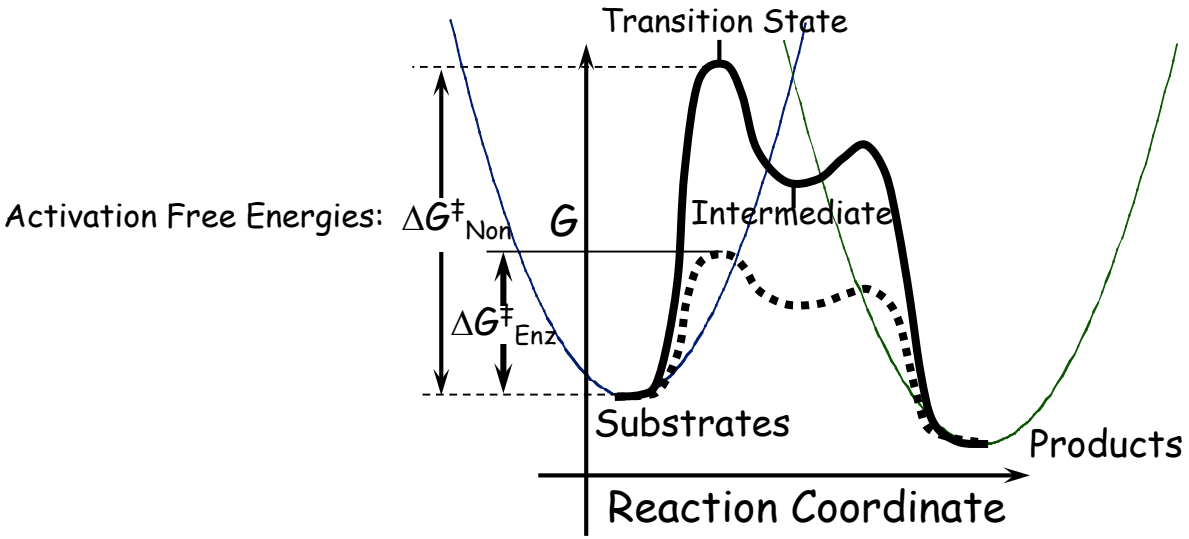
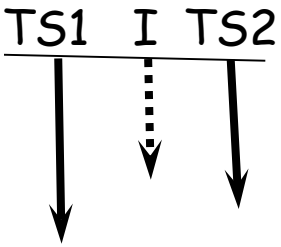
The fundamental model for enzyme catalysis: Pauling to Wolfenden

- The enzymatic rate acceleration is $[k_{cat}/(K_M*k_{non})]$, where:
  - k_{non} is the rate constant for reaction in neutral solution in the absence of enzyme,
  - k_{cat}/K_M is the apparent 2nd-order rate constant.
- $[k_{cat}/(K_M*k_{non})]$ is formally equivalent to the equilibrium binding affinity of the enzyme for the *altered substrate in the transition state*.
- This presupposes an equilibrium in solution between substrate and transition state.
- ***A stable analog of the TS will bind more tightly than substrates to the enzyme.***

Rate acceleration involves changes in *equilibria*

- The reference frame for studying enzymes involves *Free energy* vs *Reaction Coordinate*





Enzymes bind Transition states
More tightly than they bind
Other species along the reaction

- We're interested in the relative *concentrations* of:
  - [Transition state]
  - [Reactants]

$$Keq = \frac{[TransitionState]}{[Substrate]} << 1.0 = \exp\left[\frac{-\Delta G^{\ddagger}}{RT}\right] = \frac{k_1}{k_{-1}}$$

- Catalysis involves *stabilization of the Transition State*

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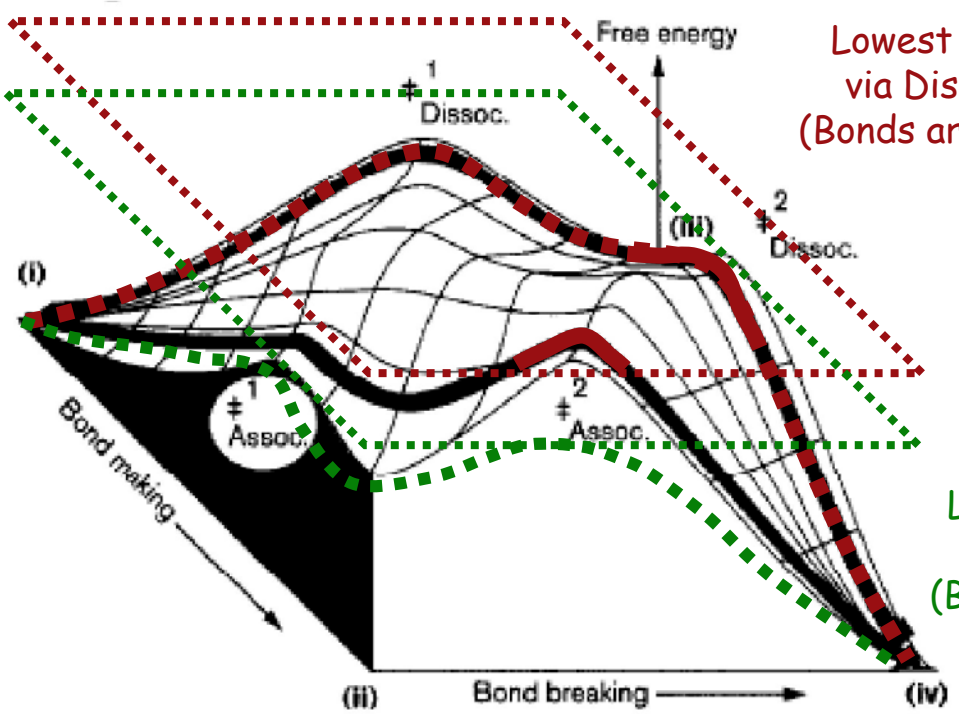
The actual transition state (and hence, mechanism) depends on the actual path between reactants and products



Lowest energy path is via Dissociative TS: (Bonds are mostly broken)

Lowest energy path is via Associative TS (Bonds are mostly made)

Small changes in the energy landscape can alter mechanism

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Limitations on rates: The Haldane Equation

- An equilibrium constant must equal the ratio of forward to reverse rate constants.
- For an enzyme catalyzed reaction, these rates are given by the second-order rate constants, k_{cat}/KM.
- Thus,

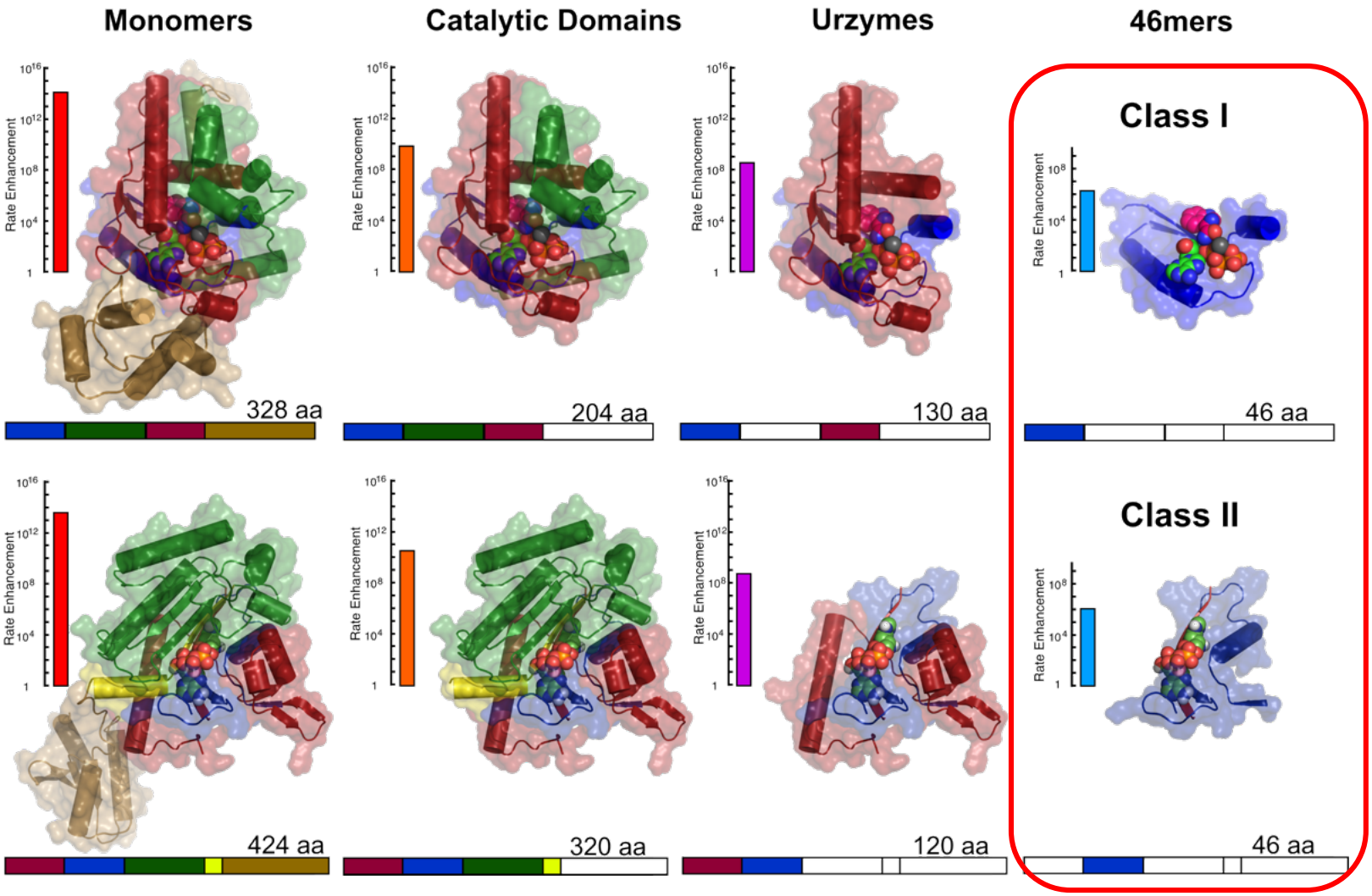
$$Keq = \frac{\left(\frac{k_{cat}}{Km}\right)_{forward}}{\left(\frac{k_{cat}}{Km}\right)_{reverse}}$$

- Example: if an enzyme catalyzes a thermodynamically unfavorable reaction at the diffusion-controlled limit, it must catalyze the reverse direction at a much lower rate.

What do enzymes bring to the table?

- Provision of specific functional groups
  - Nucleophiles
  - General Acids
  - General Bases
- Desolvation - generally less water near TS
- Electostatic stabilization
- Substrate *destabilization*
- Restricted orientation of multiple substrates (reduces $\Delta S^{\ddagger}$)
- Concerted motion complementary to the reaction coordinate
- Examples will include two hydrolases
- Next: the more complicated case of phosphoryl-transfer

Experimental deconstruction and parallel evolution of Class I, II aminoacyl-tRNA synthetases



Martinez L, Jimenez-Rodriguez M, Gonzalez-Rivera K, et al. 2015. *J. Biol. Chem.* In Press

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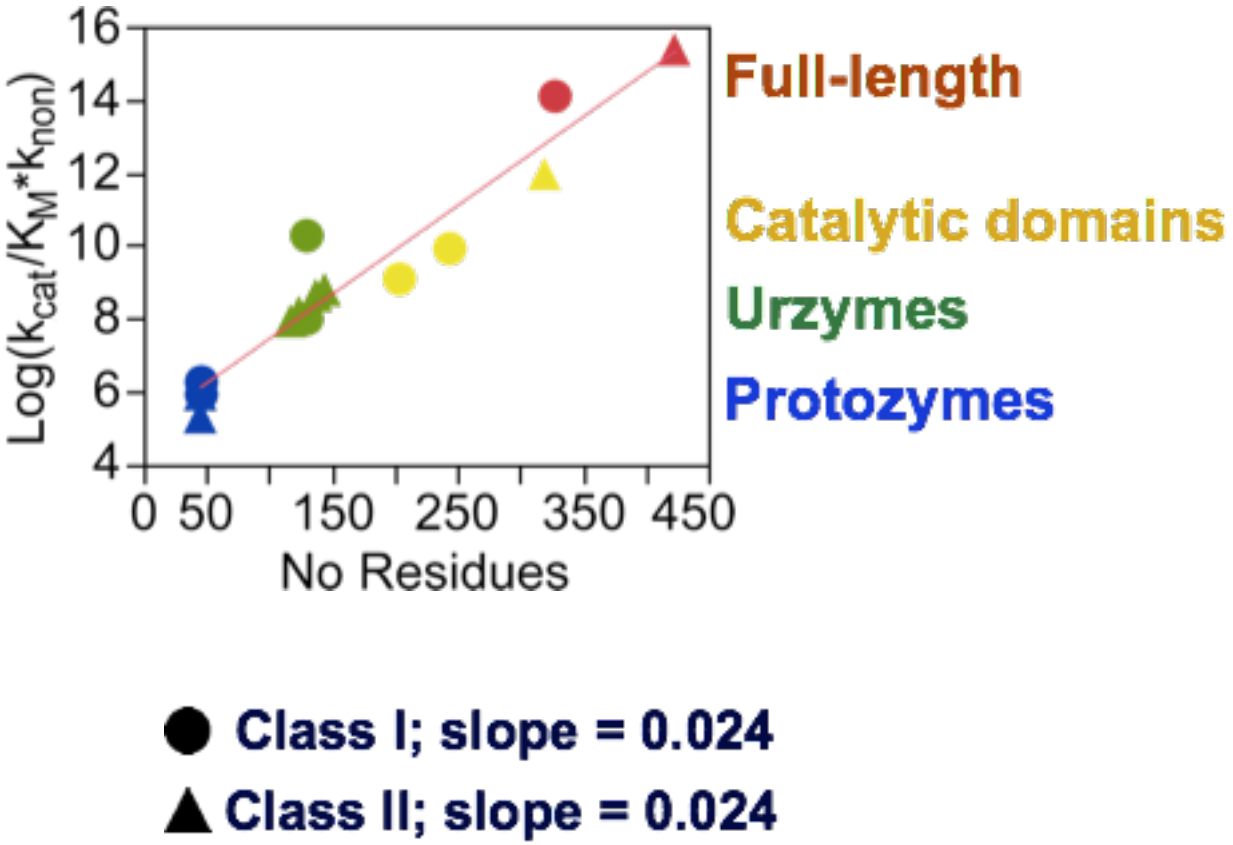
Catalytic proficiency increases linearly with Enzyme mass



● Class I; slope = 0.024

▲ Class II; slope = 0.024

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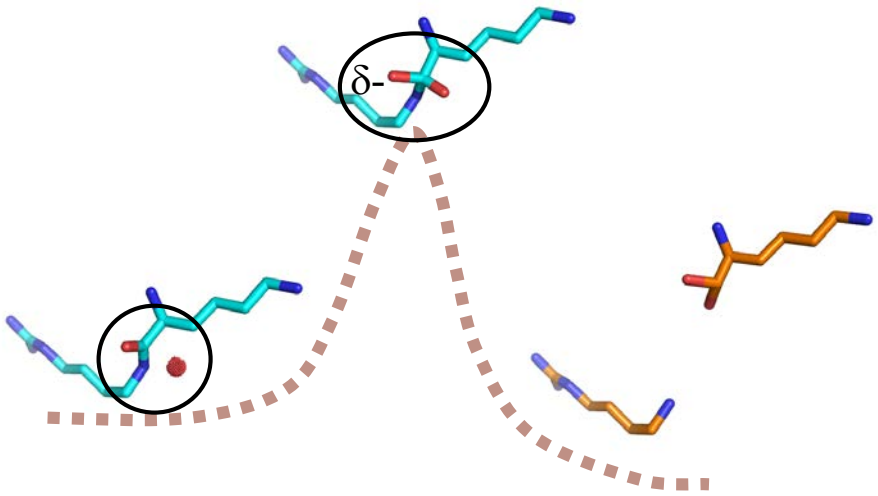
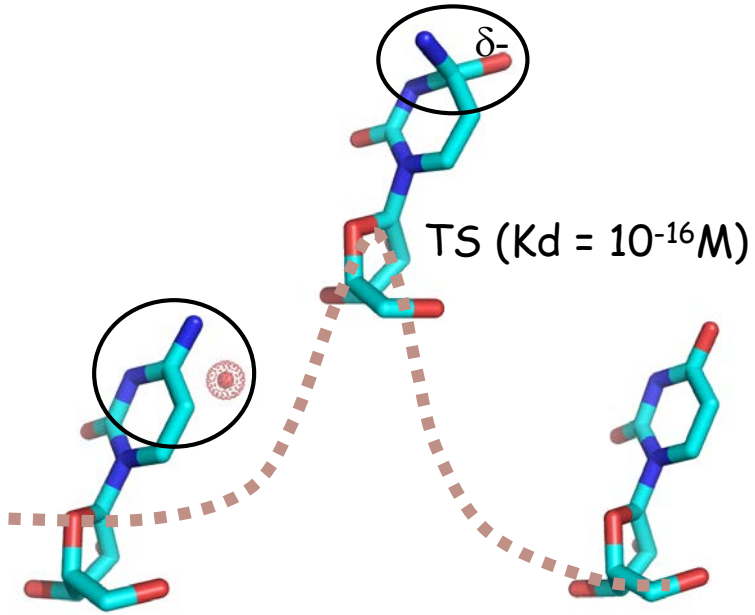
Hydrolytic reactions involve sp2 -> sp3 hybridization and generate an oxyanion

Peptide hydrolysis



Cytidine deamination



Cytidine (Kd = 10^{-5}M)

Uridine (Kd = 10^{-3}M)

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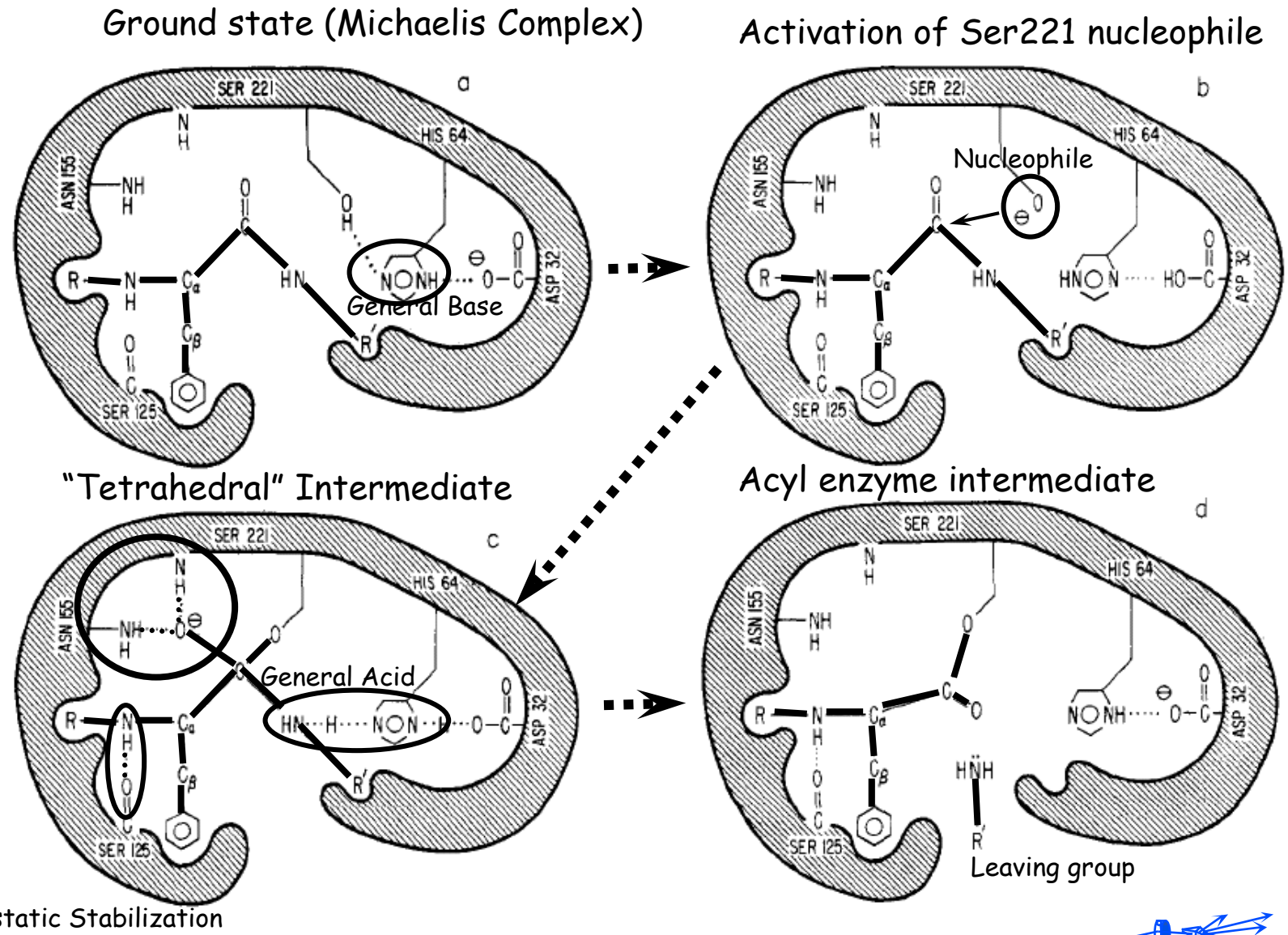
Differential binding in TS complex: Subtilisin



Electrostatic Stabilization

Robertus, et al. (1972) *Biochemistry* **11**:4293-4303

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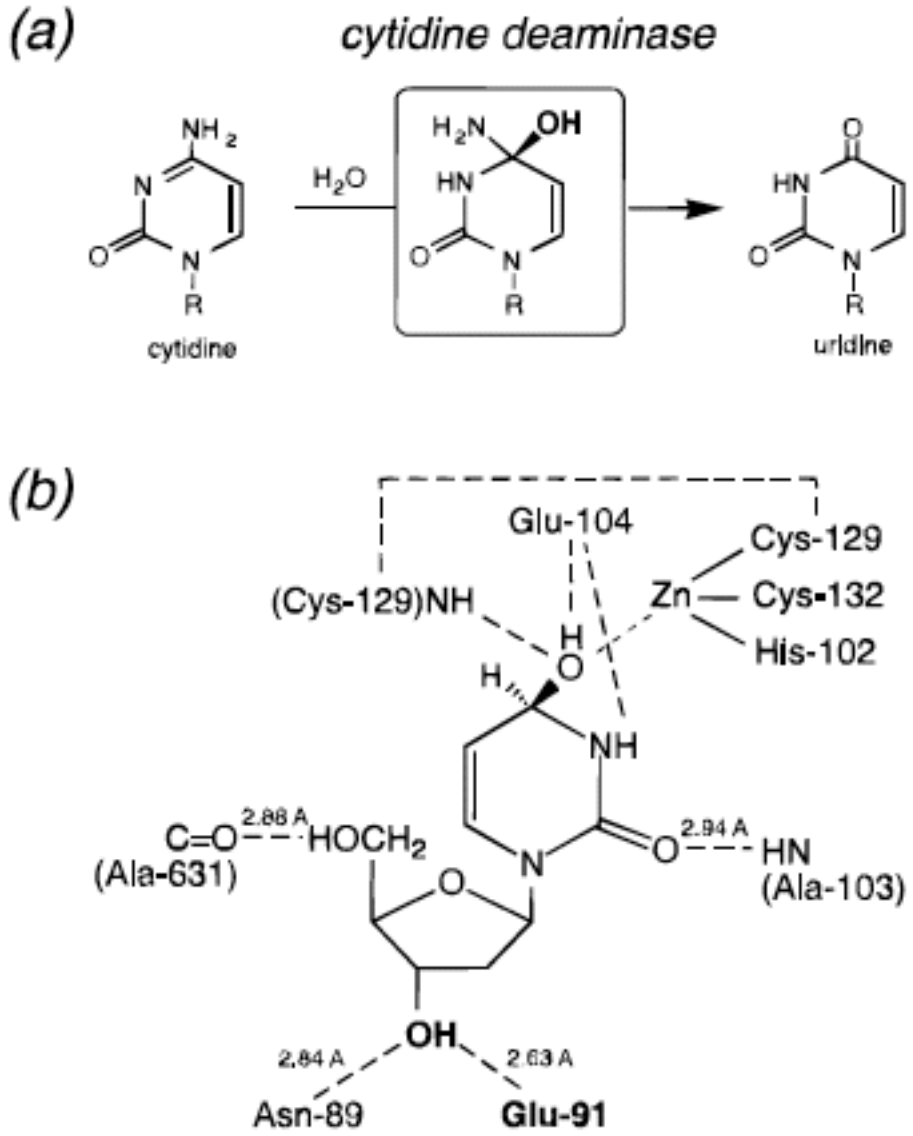
Directed mutagenesis of the subtilisin charge-relay system

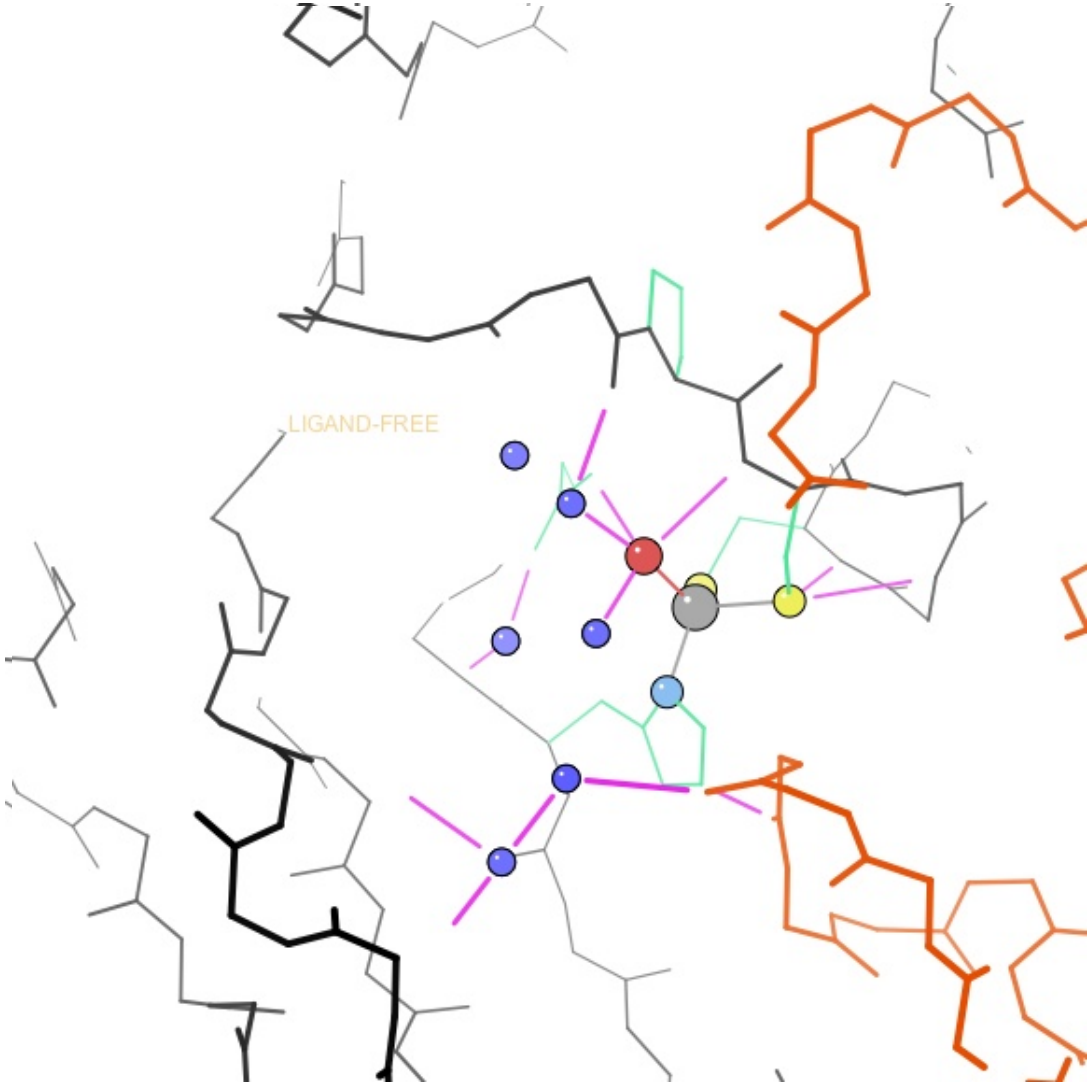
Table 1 Kinetic parameters of mutant subtilisins with the substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide at *p*H 8.60

Enzyme	Active site configuration Ser221	His64	Asp32	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$)	$\frac{k_{cat}(\text{mutant})}{k_{cat}(\text{S24C})}$
Wild type	+	+	+	$(4.4 \pm 0.1) \times 10^{1}$	180 ± 10	$(2.5 \pm 0.1) \times 10^{5}$	0.74 ± 0.01
S24C	+	+	+	$(5.9 \pm 0.2) \times 10^{1}$	220 ± 20	$(2.7 \pm 0.2) \times 10^{5}$	1
S24C:S221A	−	+	+	$(3.4 \pm 0.1) \times 10^{-5}$	420 ± 40	$(8.2 \pm 0.6) \times 10^{-2}$	$(5.8 \pm 0.1) \times 10^{-7}$
S24C:H64A	+	−	+	$(3.8 \pm 0.2) \times 10^{-5}$	390 ± 50	$(9.6 \pm 1.0) \times 10^{-2}$	$(6.4 \pm 0.2) \times 10^{-7}$
S24C:D32A	+	+	−	$(2.3 \pm 0.2) \times 10^{-3}$	480 ± 80	4.7 ± 0.7	$(3.8 \pm 0.2) \times 10^{-5}$
S24C:D32A:H64A	+	−	−	$(2.6 \pm 0.1) \times 10^{-4}$	270 ± 50	$(9.4 \pm 1.6) \times 10^{-1}$	$(4.3 \pm 0.1) \times 10^{-6}$
S24C:H64A:S221A	−	−	+	$(2.8 \pm 0.2) \times 10^{-5}$	290 ± 40	$(9.6 \pm 1.3) \times 10^{-2}$	$(4.8 \pm 0.2) \times 10^{-7}$
S24C:D32A:S221A	−	+	−	$(2.8 \pm 0.1) \times 10^{-5}$	310 ± 40	$(9.2 \pm 0.9) \times 10^{-2}$	$(4.8 \pm 0.1) \times 10^{-7}$
S24C:D32A:H64A:S221A	−	−	−	$(3.0 \pm 0.1) \times 10^{-5}$	230 ± 20	$(1.3 \pm 0.1) \times 10^{-1}$	$(5.1 \pm 0.1) \times 10^{-7}$
				k_{buffer} (s^{-1})			$\frac{k_{buffer}}{k_{cat}(\text{S24C})}$
No enzyme		none		$(1.1 \pm 0.1) \times 10^{-8}$	–	–	$(1.9 \pm 0.1) \times 10^{-10}$

Carter, P. & Wells, J. (1988) *Nature* **332**, 564-568



Cytidine Deaminase Structural Reaction Profile



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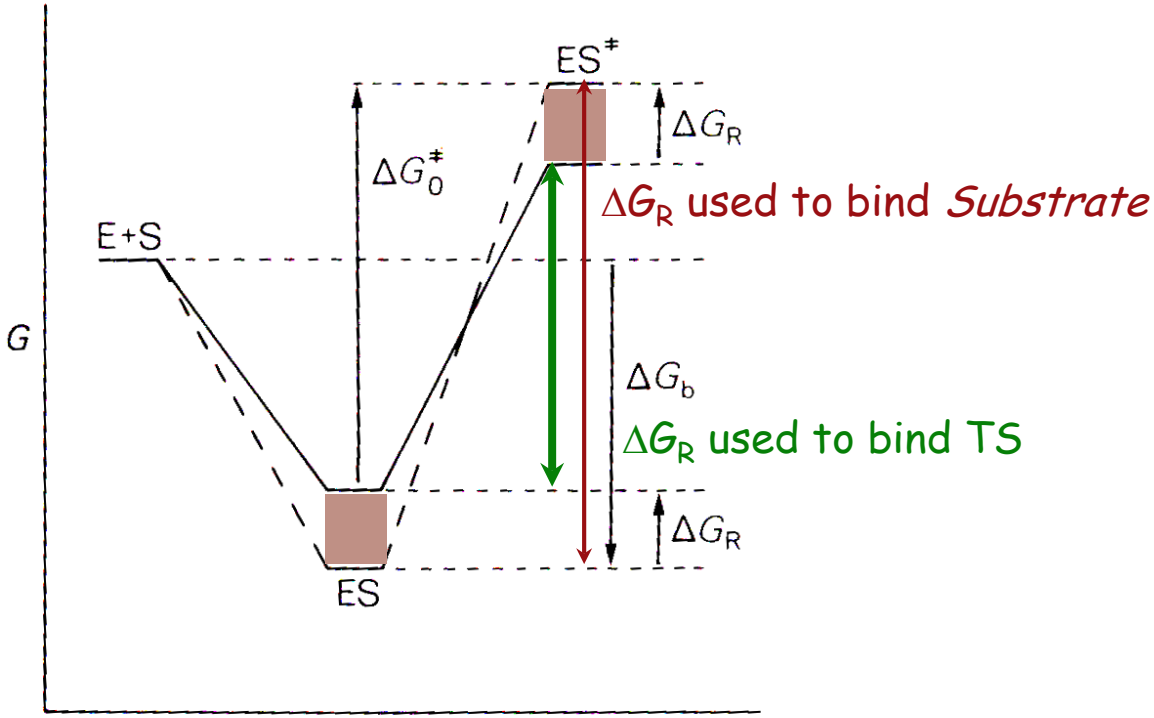
ΔG_R and transition-state stabilization

- **New interactions develop in the transition state that are absent in the ground state complexes**:
  - **Subtilisin**:
    - Hydrogen bonds from the backbone NHs to the oxyanion
    - The S1-P1 hydrogen bond
    - Penetration of the specificity residue deeper into its pocket
  - **Cytidine deaminase**:
    - The oxygen nucleophile (hydroxyl) is essentially covalently bonded at C4
    - Hydrogen bonds to O2, N3, O4, and to the ribose hydroxyl groups shorten.
    - E104 protonates the pyrimidine at N3.
- These examples illustrate *differential binding affinity*, in which *potential interactions are not made until the transition state configuration*.
- Fersht has referred to this differential with a special term, ΔG_R.

ΔG_R (Fersht, p. 354-355, Fig. 12.4)

Review: Substrate and transition state have different structures; enzymes can bind them with different affinities.



Activation free energy for k_{cat} depends on where along the rx coord ΔG_R is realized; when used to bind TS more tightly than Michaelis complex it contributes twice.

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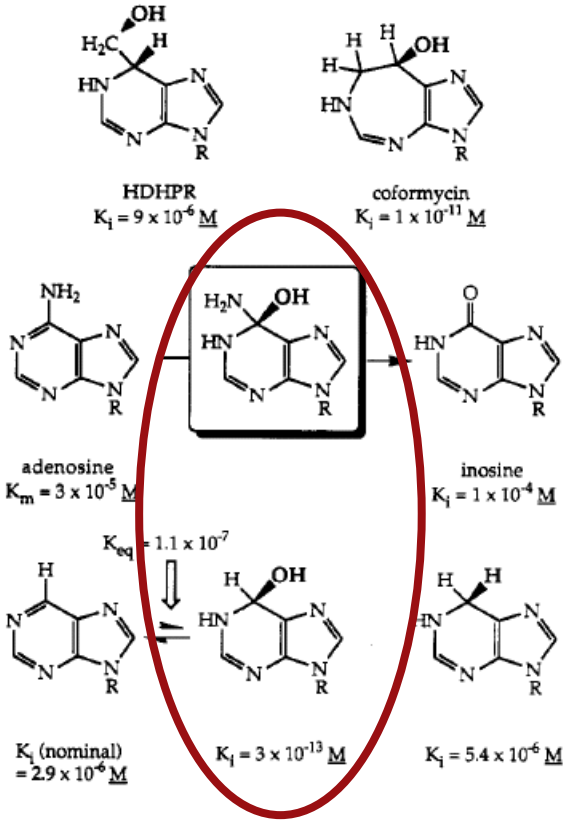
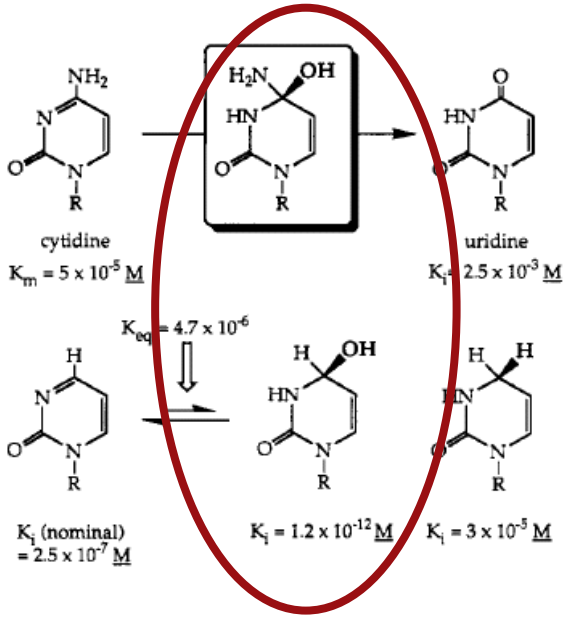
Transition state analog inhibitors bind much more tightly than substrates





Wolfenden & Kati (1991) *Acc. Chem. Res.*,**24**, 209-215

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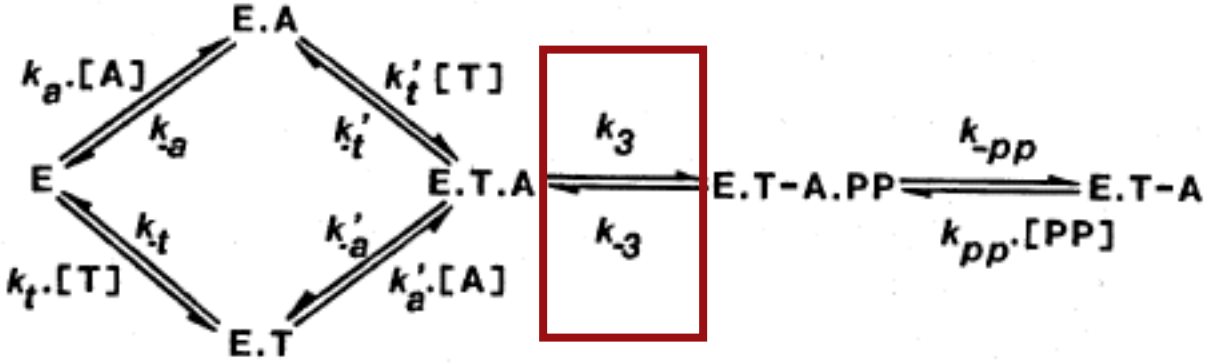
Pre-steady state kinetics and mechanism



SCHEME 1

Table 1. Presteady state kinetic parameters for formation of tyrosyl adenylate

Enzyme	k_3,* s^{-1}	K_S for tyrosine, μM	K_S for ATP, mM
Tyrosyl-tRNA synthetase†	38	12	4.7
Tyrosyl-tRNA synthetase(His-45 → Gly-45)	0.16	10	1.2
Tyrosyl-tRNA synthetase(Thr-40 → Ala-40)	0.0055	8.0	3.8
Tyrosyl-tRNA synthetase(Thr-40 → Ala-40; His-45 → Gly-45)	0.00012	4.5	1.1

Experiments were performed at 25°C at pH 7.8 (144 mM Tris·HCl) in the presence of 10 mM $MgCl_2$ (free), 1 unit of inorganic pyrophosphatase per ml, 14 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride under presteady state conditions. k_3 is the forward rate constant for the formation of tyrosyl adenylate (Scheme 1); K_S is the dissociation constant for the substrate ($= k'_{-t}/k'_t$ or k'_{-a}/k'_a for tyrosine and ATP, respectively).

*Extrapolated to infinite substrate concentrations.

†From Wells and Fersht (14). The value of K_S for tyrosine of wild-type enzyme was obtained from equilibrium dialysis and equals k_{-t}/k_t.

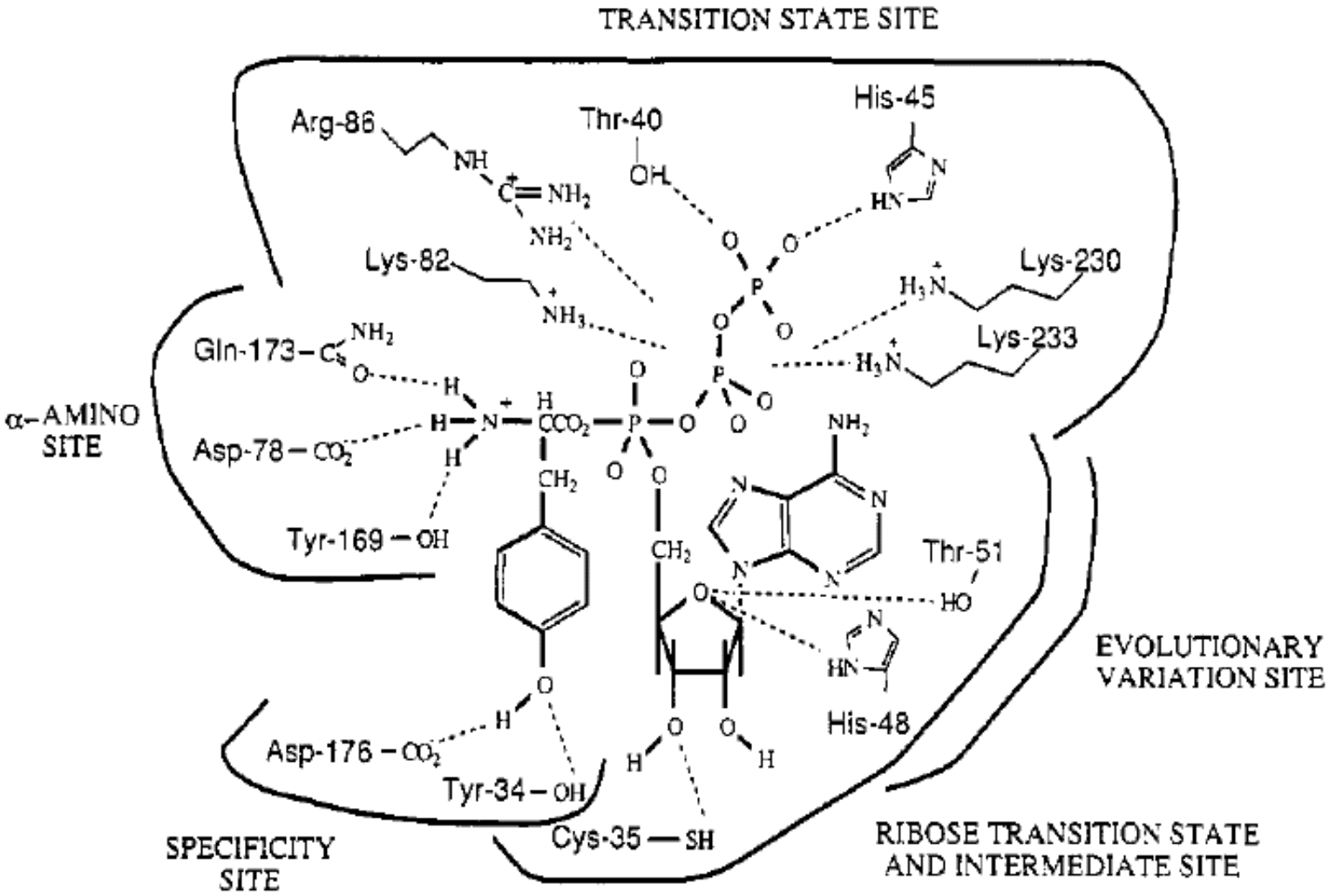


Table I: Interaction Energies of Side Chains of Tyrosyl-tRNA Synthetase with Reagents

residue	interaction energy of side chains[a] in complex with Tyr	ATP	[Tyr-ATP]‡	PP_i	Tyr-AMP
		Tyrosine Binding Site			
Tyr-34	+	0	+	0	+
Asp-78	++++	++*	++++	++*	++++
Tyr-169	++++	0	++++	0	++++
Gln-173	++++	++*	++++	+*	++++
		Nucleotide and Pyrophosphate Site			
Cys-35	0	0	++	0	+++
Thr-40	0	0	++++	++++	0
His-45	0	0	++++	++++	0
His-48	0	0	+++	0	+++
Thr-51	0	0	0	0	–
Lys-82	0	++	++++	++++	0
Arg-86	0	0	++++	++++	–
Asp-194	0	0	++++	+	+++
Lys-230	0	0	++++	++++	0
Lys-233	0	++++	++++	++++	0

[a] Apparent stabilization energy from the side chain in kcal/mol: 0 = −0.5 to +0.5; + = 0.5–1.0; ++ = 1.0–1.5; +++ = 1.5–2.0; ++++ = >2.0; − = −0.5 to −1.0; * = evidence for some disruption of protein structure on mutation.

Tryptophan +ATP <=> Tryptophanyl-AMP + PPi

X-ray structures

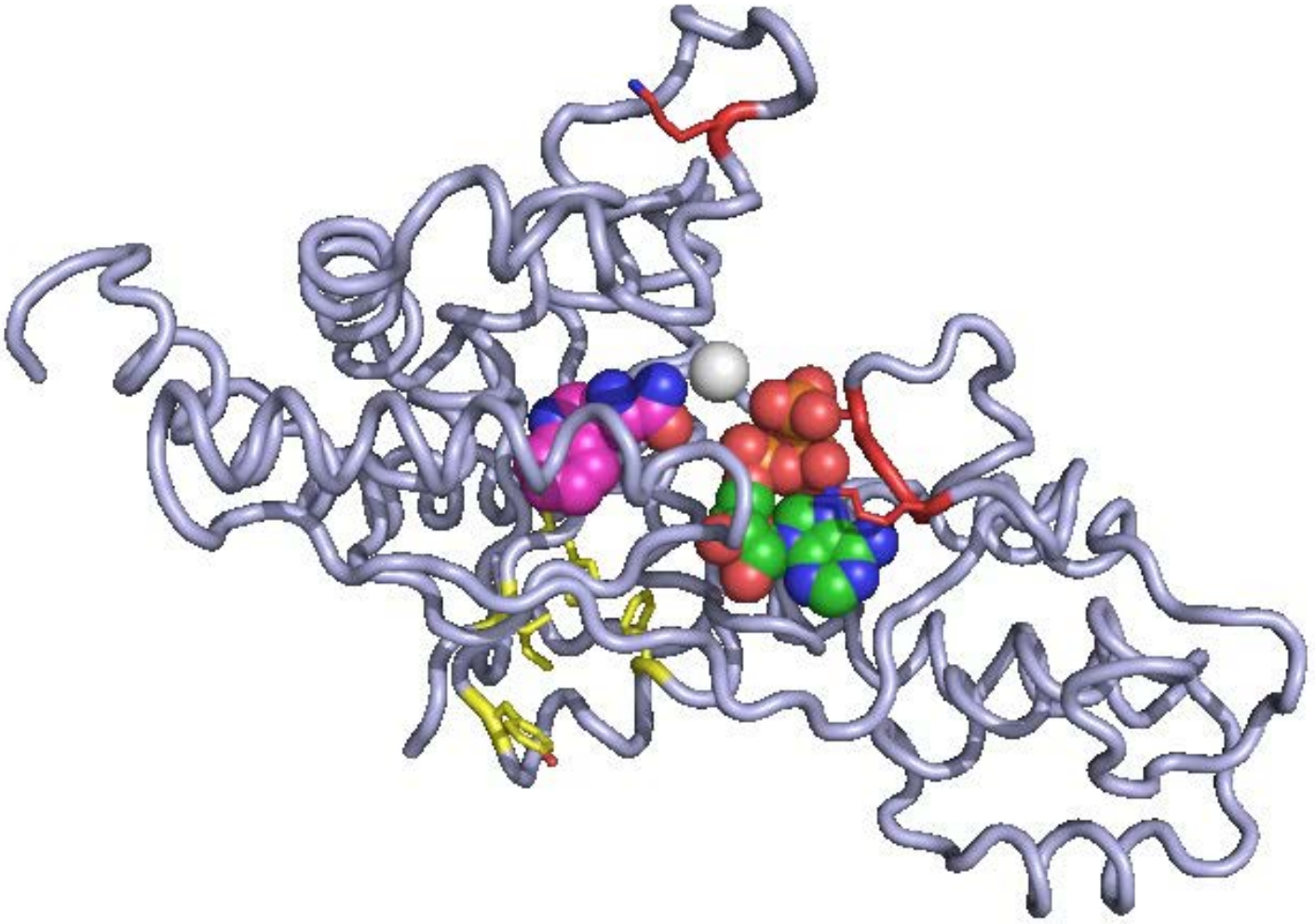
OPEN

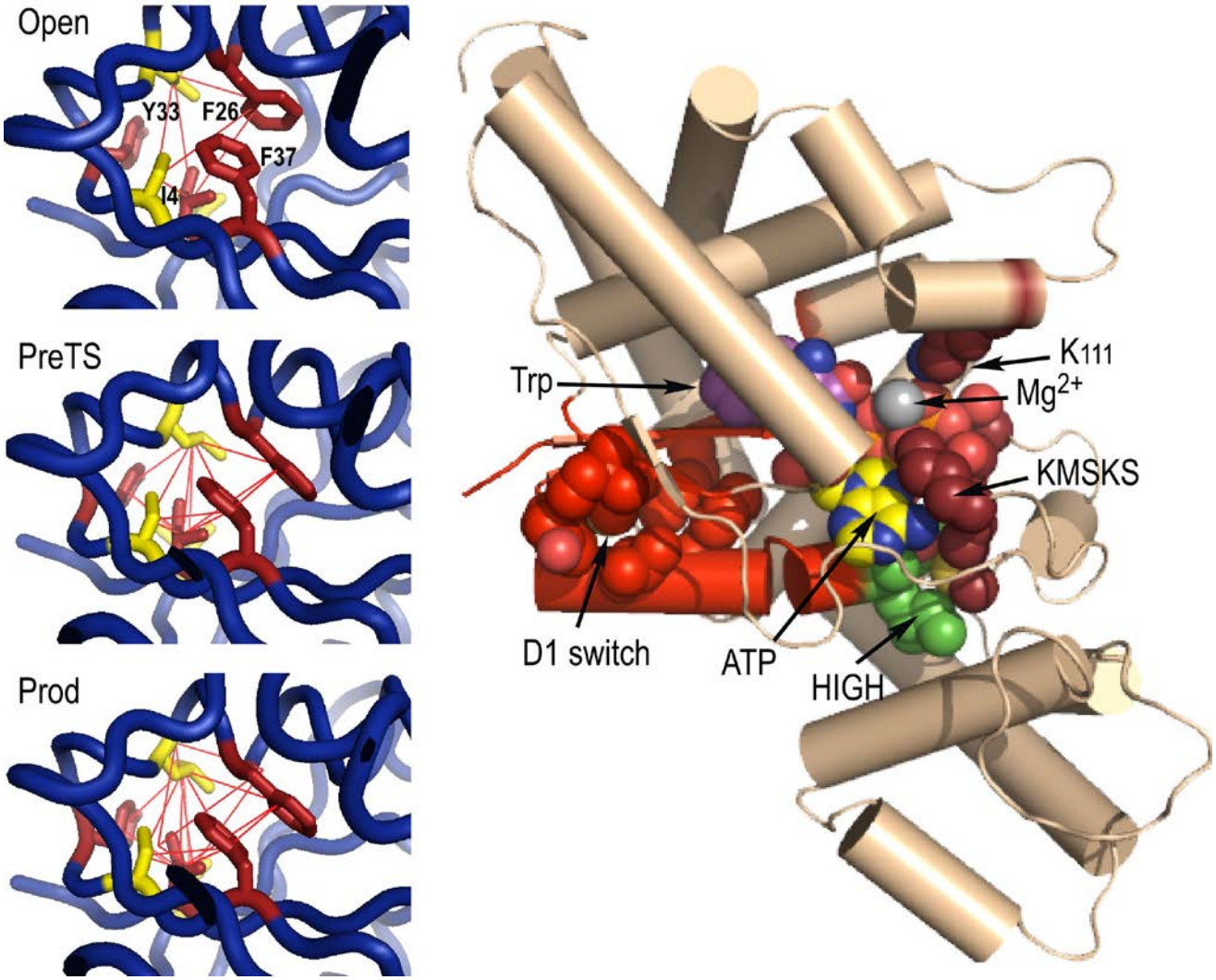
Induced-Fit

CLOSED

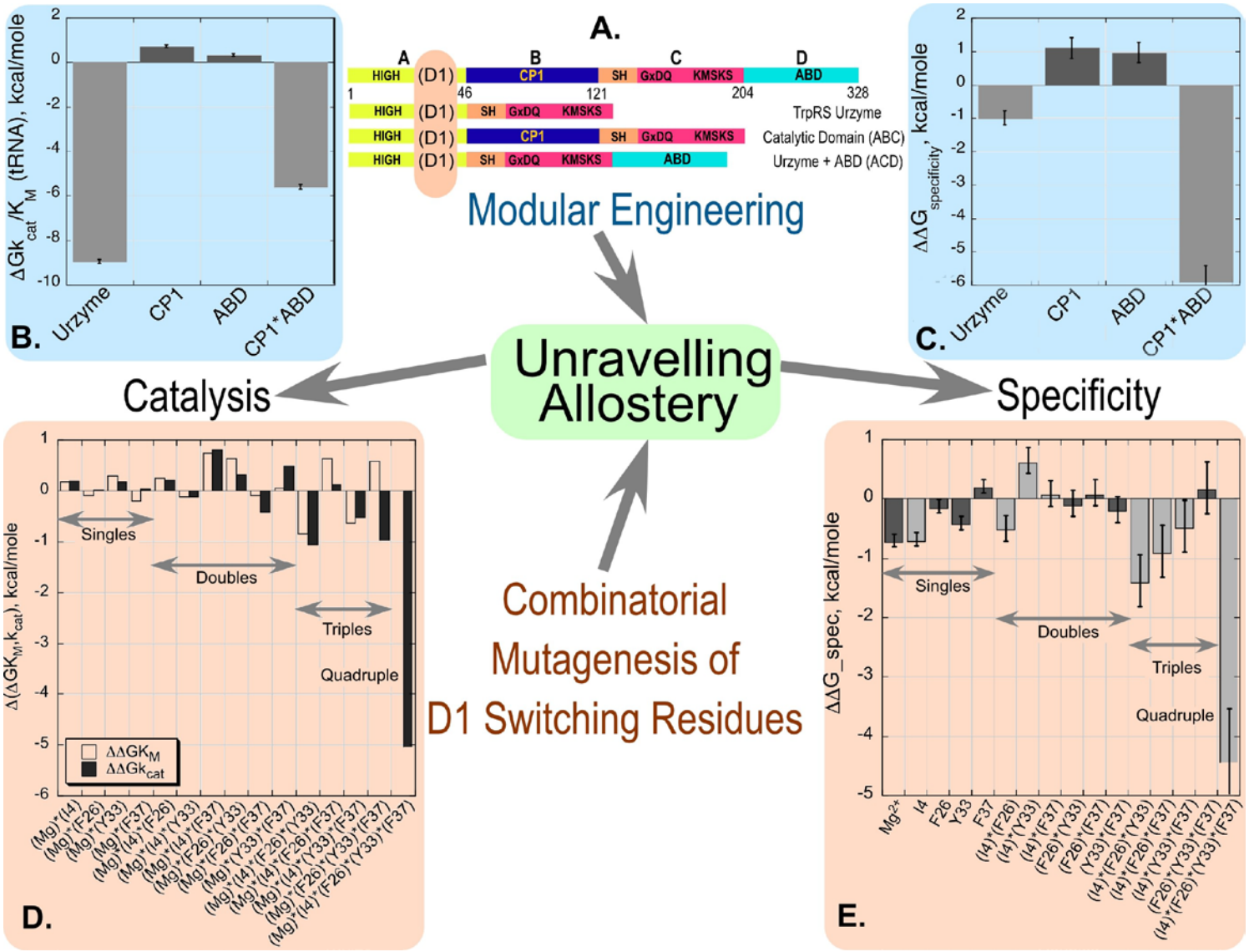
Catalysis

PRODUCT





Modular Engineering, Combinatorial mutagenesis agreel



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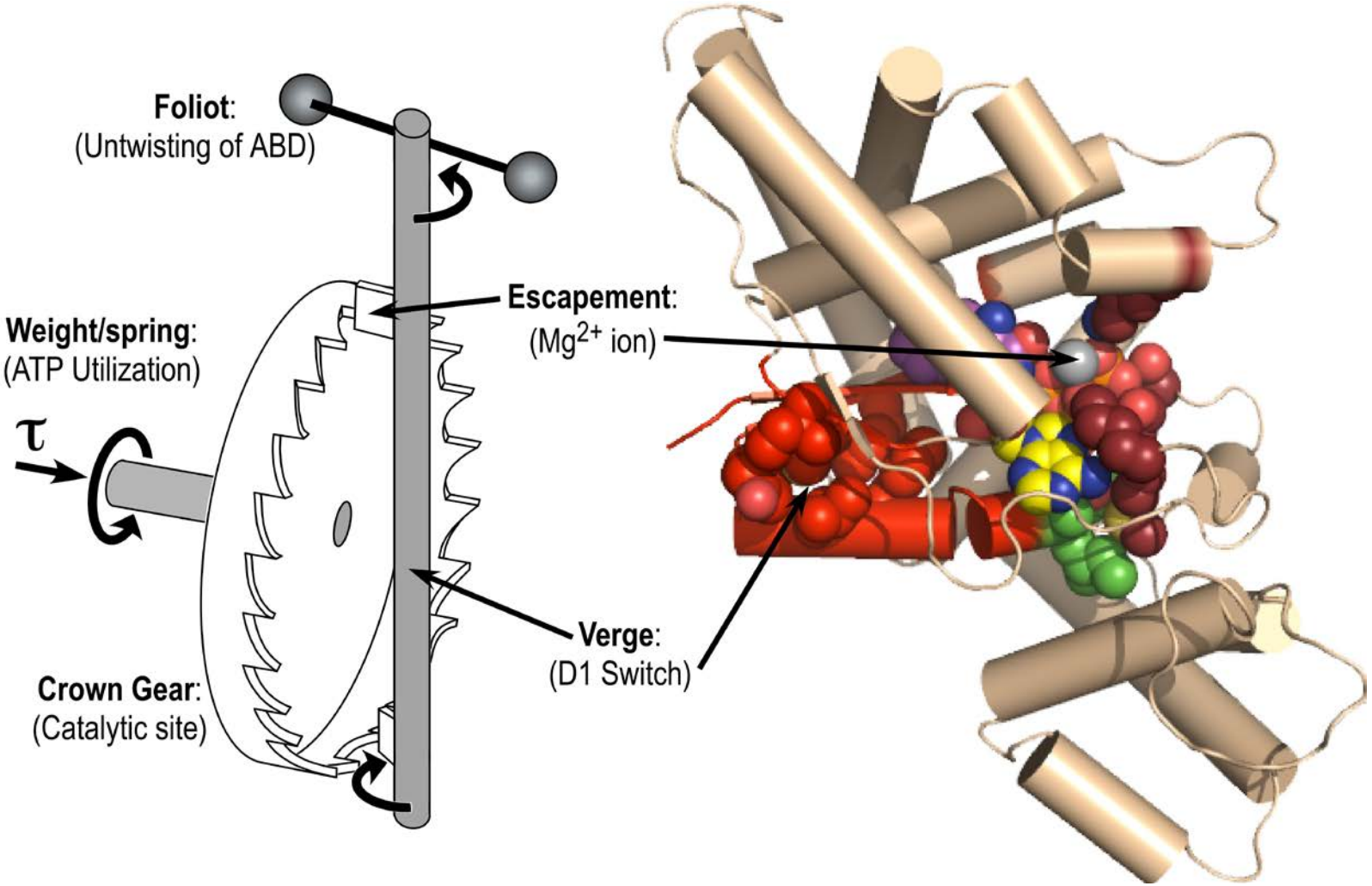
A Master Switch couples domain movement to catalysis



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Key Ideas About Catalysis

- Chemistry is slow in water and in the cold.
- Rates depend in the [TS]; Activation Free Energy, $\Delta G^{\ddagger}$
- Saturation Kinetics imply a Steady State (kcat/Km).
- Equilibria between E and TS determines the rate acceleration.
- Enzymes use a variety of strategies to specifically bind the TS.
- Analogs of the transition state structure bind very tightly.
- Mutagenesis, Pre-Steady State Kinetics test mechanisms.

Study Questions

1. **Uncatalyzed reactions occur faster at higher temperatures. A factor of two is often observed for every 10^{o} hotter it gets. How much faster were all chemical reactions necessary for life when the earth cooled sufficiently for liquid water to form?**

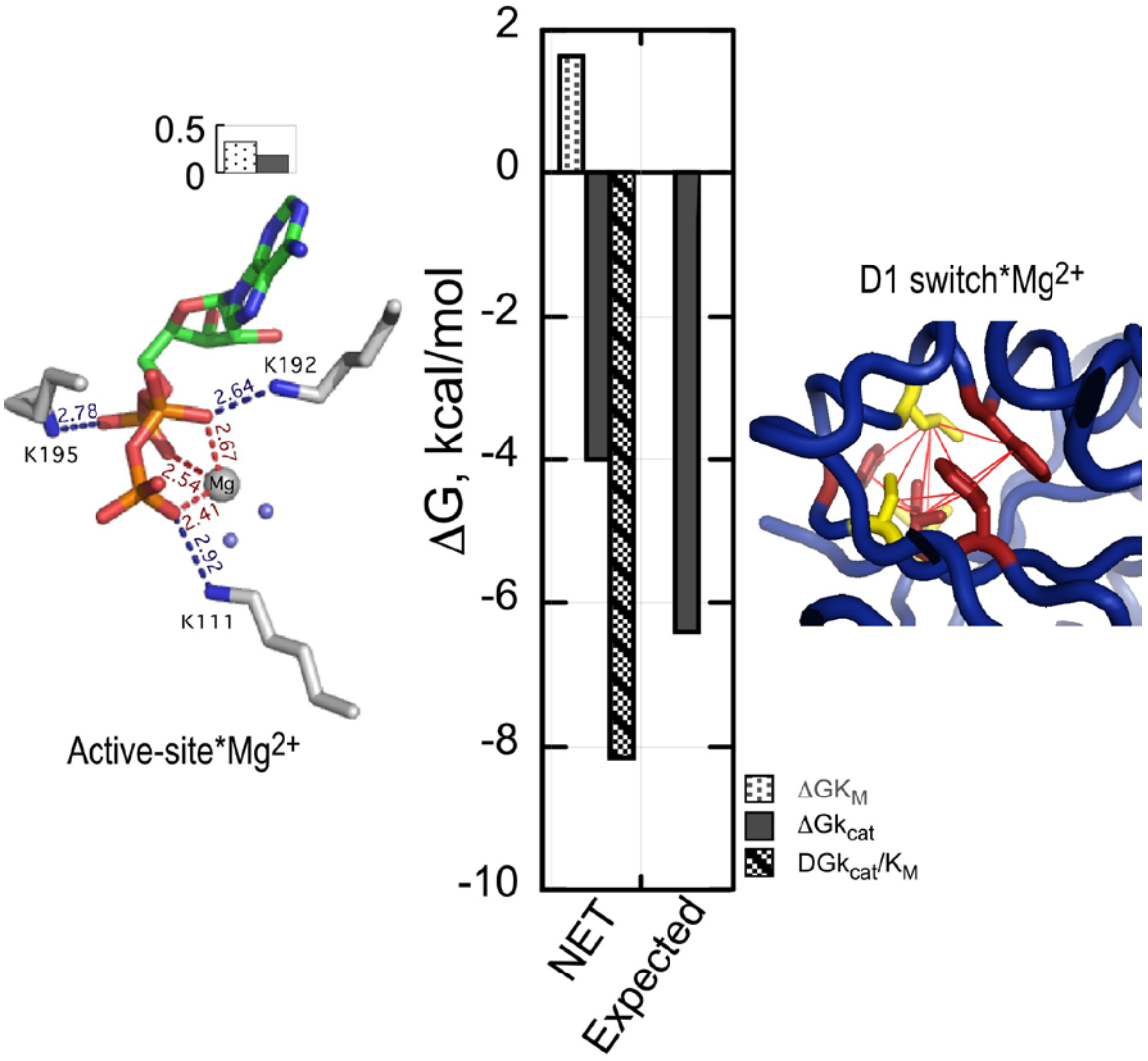
2. **Triose phosphate isomerase (TIM) catalyzes the (reversible) reaction of Dihydroxyacetone phosphate (DHAP) to Glyceraldehyde 3 Phosphate (G3P). The equilibrium for the reaction, DHAP => G3P is 1.8 Kcal/mol at 25°C. The Km values for DHAP and G3P are 870 mM and 460 mM, respectively. The turnover number for the (reverse) reaction of G3p => DHAP is 4.3 x 10^{3}/sec. What is the k_{cat} value for the reaction DHAP => G3P.**

3. **What is the most general factor determining the overall rate enhancement of an enzyme, ie., its proficiency, k_{cat}/KM, relative to k_{Non}?**

4. **Mutation of active site residues for several enzymes has revealed that the sum of contributions of all mutated residues, ($\Sigma(\Delta\Delta G^{\ddagger})$ is substantially greater than the total rate acceleration ($\Delta G^{\ddagger}$). How can this be true?**

5. **A near Guinness record holder for the most proficient enzyme, ODCase, accelerates orotidylate decarboxylation by 15 orders of magnitude just by eliminating water from the environment of the reaction. Discuss why water prefers reactions and products to transition states in general.**

Long-range dynamic interactions activate Mg^{2+} ion



B.

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Ed Taylor: energy transduction revealed

Myosin vs ***Actomyosin***

$$AM + ATP \rightleftharpoons A \cdot M \cdot ATP \xrightarrow{20\ sec^{-1}} AM + ADP + P$$

$$+A \uparrow \downarrow -A$$

$$M + ATP \rightleftharpoons M \cdot ATP \xrightarrow{0.1\ sec^{-1}} M + ADP + P$$

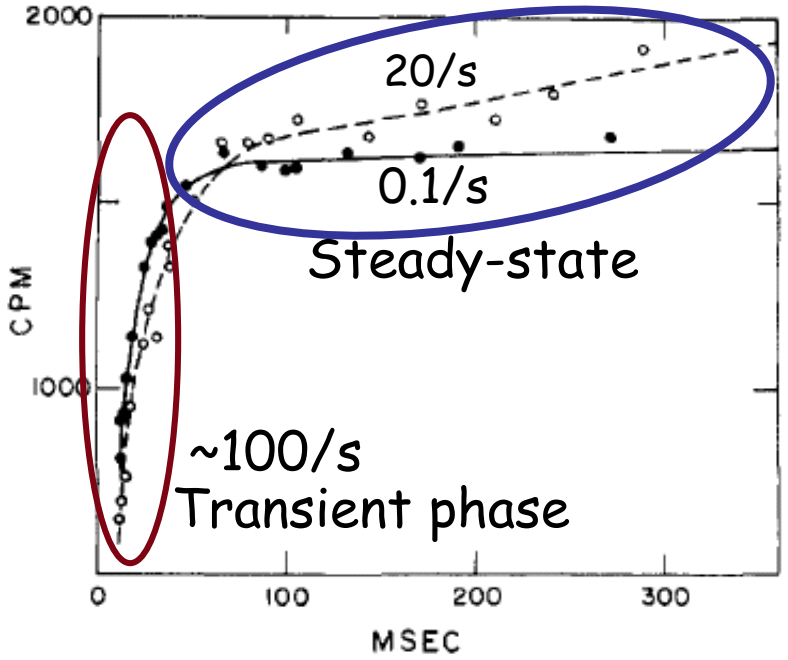

FIGURE 1: Early phase in the hydrolysis of ATP by HMM and acto-HMM. (●) Phosphate liberation by HMM, (○) phosphate liberation by acto-HMM. Conditions 0.05 M KCl–0.02 M Tris buffer (pH 8), 20°, 5×10^{-3} M $MgCl_2$, 25 mM ATP, 4.8 moles of actin/mole of HMM, and 1 mg/ml of HMM. Intercept of the linear portion corresponds to an early burst of approximately 1.1 moles of phosphate/mole of HMM.

Enzymes' most important purpose is synchronization!

Directed mutagenesis of Cytidine Deaminase

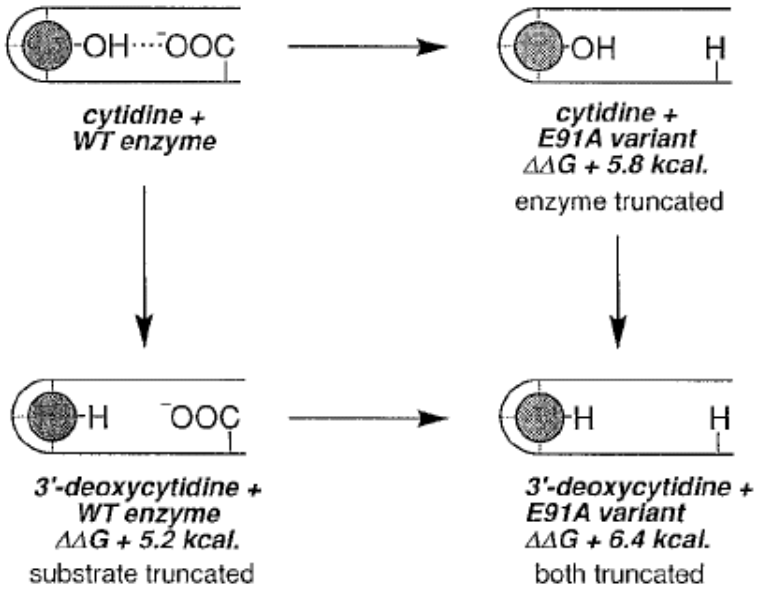

Table 1: Kinetic Parameters of Wild-Type and E91A Cytidine Deaminases[a]

	substrate	
	cytidine	3′-deoxycytidine
	wild-type enzyme	
K_m (mM)	0.120	2.0
k_{cat} (s^{-1})	299	0.8
k_{cat}/K_m (M^{-1} s^{-1})	2.5×10^6	400
	E91A enzyme	
K_m (mM)	62	13
k_{cat} (s^{-1})	9.3	0.6
k_{cat}/K_m (M^{-1} s^{-1})	150	46

[a] The kinetic parameters for the wild-type enzyme with cytidine as substrate are from ref *8*. Standard errors in the present work were less than or equal to ±11% for K_m values and ±5% for k_{cat} values.

Carlow, et. al. (1998) *Biochemistry,* **37**:1197-1203

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