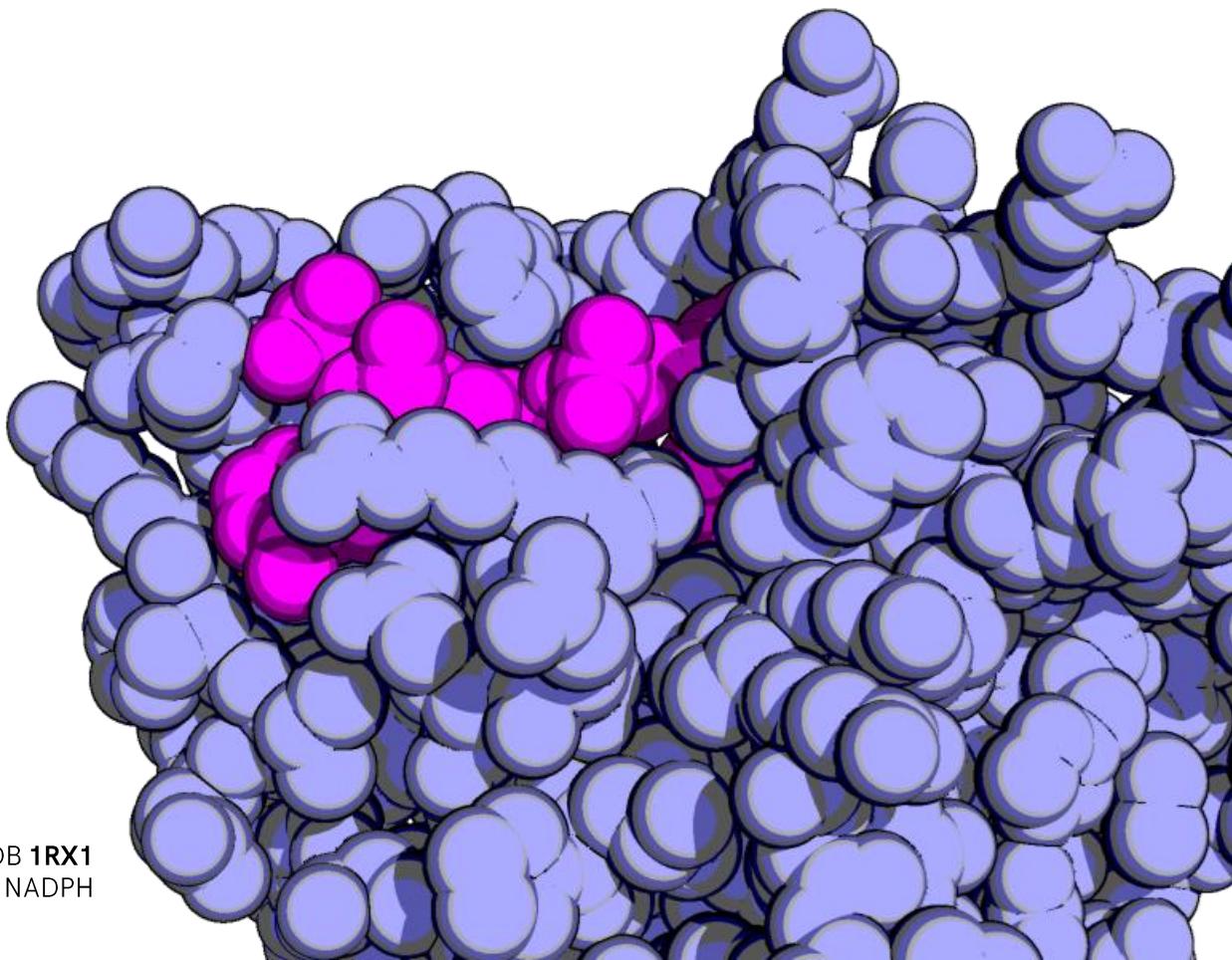


Enzyme catalysis: better, but how?

June 2023
Literature Meeting

PDB 1RX1
dihydrofolate reductase complexed with NADPH



Enzymes are good at promoting reactions

How does triosephosphate isomerase do it?

Knowles *Nature* **1991**

How do enzymes achieve accelerated rates for difficult chemical transformations?

Benkovic; Hammes-Schiffer *Science* **2003**

What is the origin of the enormous catalytic power of enzymes?

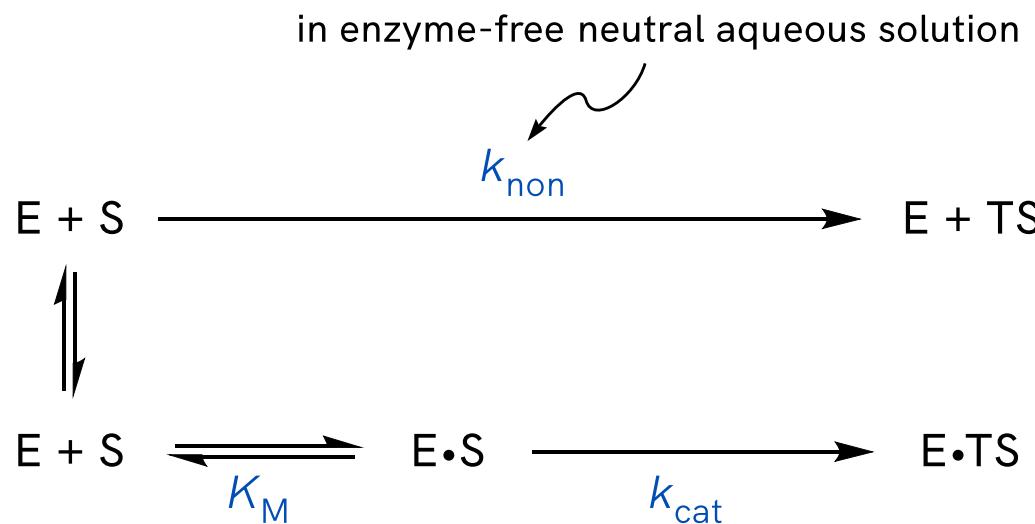
Warshel *Chem. Rev.* **2006**

What happens inside an enzyme's active site to allow slow and difficult chemical reactions to occur so rapidly?

Fried; Boxer *Annu. Rev. Biochem.* **2017**

How do enzymes work?

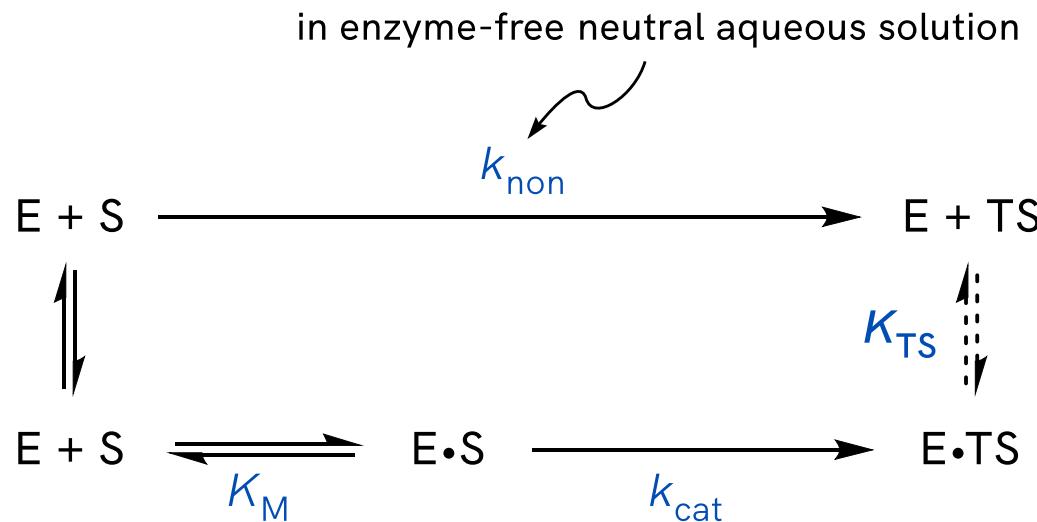
Enzymes exhibit astronomical catalytic proficiency



k_{cat}/K_M : second-order "rate constant" for enzymatic reaction

Enzyme	Nonenzymatic $t_{1/2}^*$	k_{non}^* (s^{-1})	k_{cat}^{\dagger} (s^{-1})	$k_{\text{cat}}/K_m^{\ddagger}$ ($\text{s}^{-1} \text{M}^{-1}$)	Rate enhancement ($k_{\text{cat}}/k_{\text{non}}$)	Catalytic proficiency $[(k_{\text{cat}}/K_m)/k_{\text{non}}]$ (M^{-1})
OMP decarboxylase	78,000,000 years	2.8×10^{-16}	39	5.6×10^7	1.4×10^{17}	2.0×10^{23}
Staphylococcal nuclease	130,000 years	1.7×10^{-13}	95	1.0×10^7	5.6×10^{14}	5.9×10^{19}
Adenosine deaminase	120 years	1.8×10^{-10}	370	1.4×10^7	2.1×10^{12}	7.8×10^{16}
AMP nucleosidase	69,000 years	1.0×10^{-11}	60	5.0×10^5	6.0×10^{12}	5.0×10^{16}
Cytidine deaminase	69 years	3.2×10^{-10}	299	2.9×10^6	1.2×10^{12}	9.1×10^{15}
Phosphotriesterase	2.9 years	7.5×10^{-9}	2100	4.0×10^7	2.8×10^{11}	5.3×10^{15}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	6.6×10^6	1.9×10^{11}	2.2×10^{15}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66000	3.0×10^8	3.9×10^{11}	1.8×10^{15}
Triosephosphate isomerase	1.9 days	4.3×10^{-6}	4300	2.4×10^8	1.0×10^9	5.6×10^{13}
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.1×10^6	1.9×10^6	4.2×10^{10}
Carbonic anhydrase	5 s	1.3×10^{-1}	1×10^6	1.2×10^8	7.7×10^6	9.2×10^8
Cyclophilin, human	23 s	2.8×10^{-2}	13000	1.5×10^7	4.6×10^5	5.3×10^8

Enzymes exhibit astronomical catalytic proficiency



k_{cat}/K_M : second-order "rate constant" for enzymatic reaction

Rate acceleration (or "catalytic proficiency") can be defined as

$$K_{TS} = \frac{k_{cat}/K_M}{k_{non}}$$

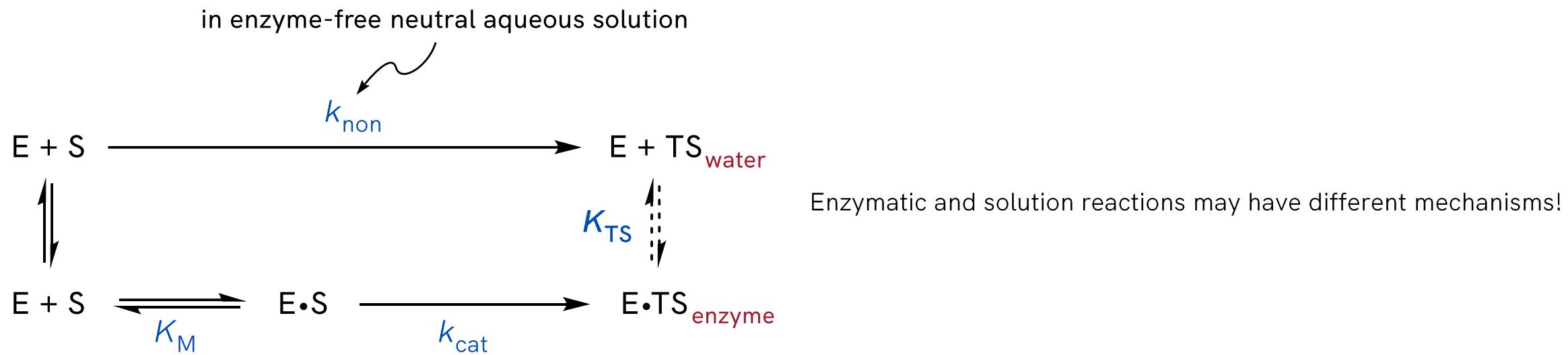
By the quasi-thermodynamic cycle, K_{TS} (units M⁻¹) turns out to be the binding constant of E to TS.

Enzyme	Nonenzymatic $t_{1/2}^*$	k_{non}^* (s^{-1})	k_{cat}^\dagger (s^{-1})	$k_{\text{cat}}^\dagger/K_m^\ddagger$ ($\text{s}^{-1} \text{M}^{-1}$)	Rate enhancement ($k_{\text{cat}}/k_{\text{non}}$)	Catalytic proficiency ($(k_{\text{cat}}/K_m)/k_{\text{non}}$ (M^{-1}))
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AMP nucleosidase	69,000 years	1.0×10^{-11}	60	5.0×10^5	6.0×10^{12}	5.0×10^{16}
Cytidine deaminase	69 years	3.2×10^{-10}	299	2.9×10^6	1.2×10^{12}	9.1×10^{15}
Phosphotriesterase	2.9 years	7.5×10^{-9}	2100	4.0×10^7	2.8×10^{11}	5.3×10^{15}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	6.6×10^6	1.9×10^{11}	2.2×10^{15}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66000	3.0×10^8	3.9×10^{11}	1.8×10^{15}
Triosephosphate isomerase	1.4 seconds	1.0×10^{-3}	1000000	1.0×10^{10}	5.6×10^{13}	
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.1×10^6	1.9×10^6	4.2×10^{10}
Carbonic anhydrase	5 s	2.6×10^{-3}	100000	1.2×10^8	7.7×10^6	9.2×10^8
Cyclophilin, human	23 s	2.8×10^{-2}	13000	1.5×10^7	4.6×10^5	5.3×10^8

What is the origin of large enzymatic catalytic proficiencies?

How do enzymes work?

Wolfenden's catalytic proficiency is imprecise



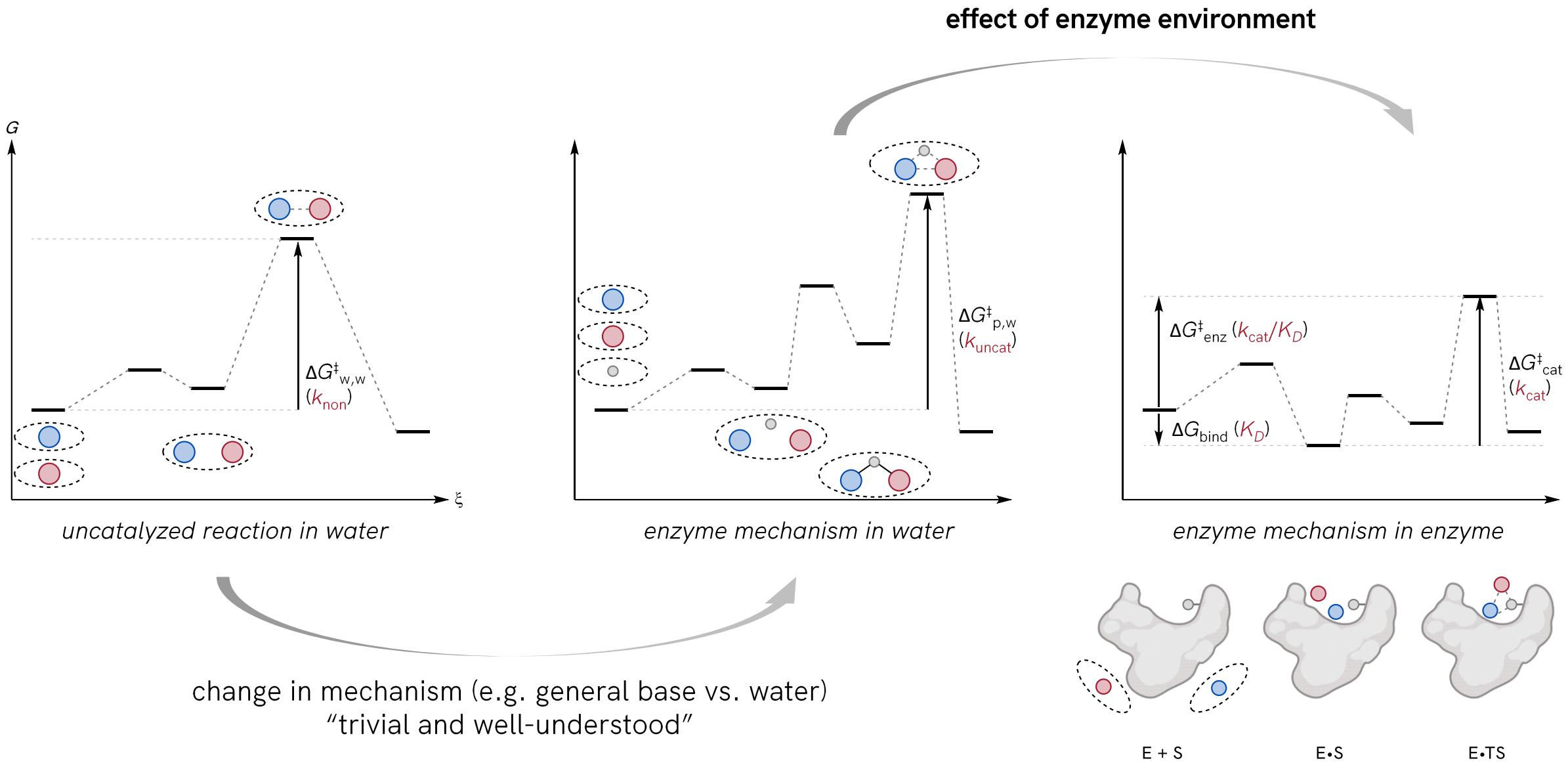
Enzyme	Nonenzymatic $t_{1/2}^*$	$k_{\text{non}}^* \text{ (s}^{-1}\text{)}$	$k_{\text{cat}}^{\dagger} \text{ (s}^{-1}\text{)}$	$k_{\text{cat}}^{\dagger}/K_m^{\dagger} \text{ (s}^{-1}\text{ M}^{-1}\text{)}$	Rate enhancement ($k_{\text{cat}}/k_{\text{non}}$)	Catalytic proficiency $[(k_{\text{cat}}/K_m)/k_{\text{non}}] \text{ (M}^{-1}\text{)}$
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Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	6.6×10^6	1.9×10^{11}	2.2×10^{15}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66000	3.0×10^8	3.9×10^{11}	1.8×10^{15}
Triosephosphate isomerase	0.24 s	1.0×10^{-4}	1000000	1.0×10^9	1.0×10^{19}	5.6×10^{13}
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.1×10^6	1.9×10^6	4.2×10^{10}
Carbonic anhydrase	5 s	2.6×10^{-5}	120000	1.2×10^8	7.7×10^6	9.2×10^8
Cyclophilin, human	23 s	2.8×10^{-2}	13000	1.5×10^7	4.6×10^5	5.3×10^8

How do enzymes promote reactions with great rate acceleration relative to the same mechanism in water?

What is the origin of large enzymatic catalytic proficiencies?

How do enzymes work?

Enzymes' catalytic power arises from the large effect of the active site environment

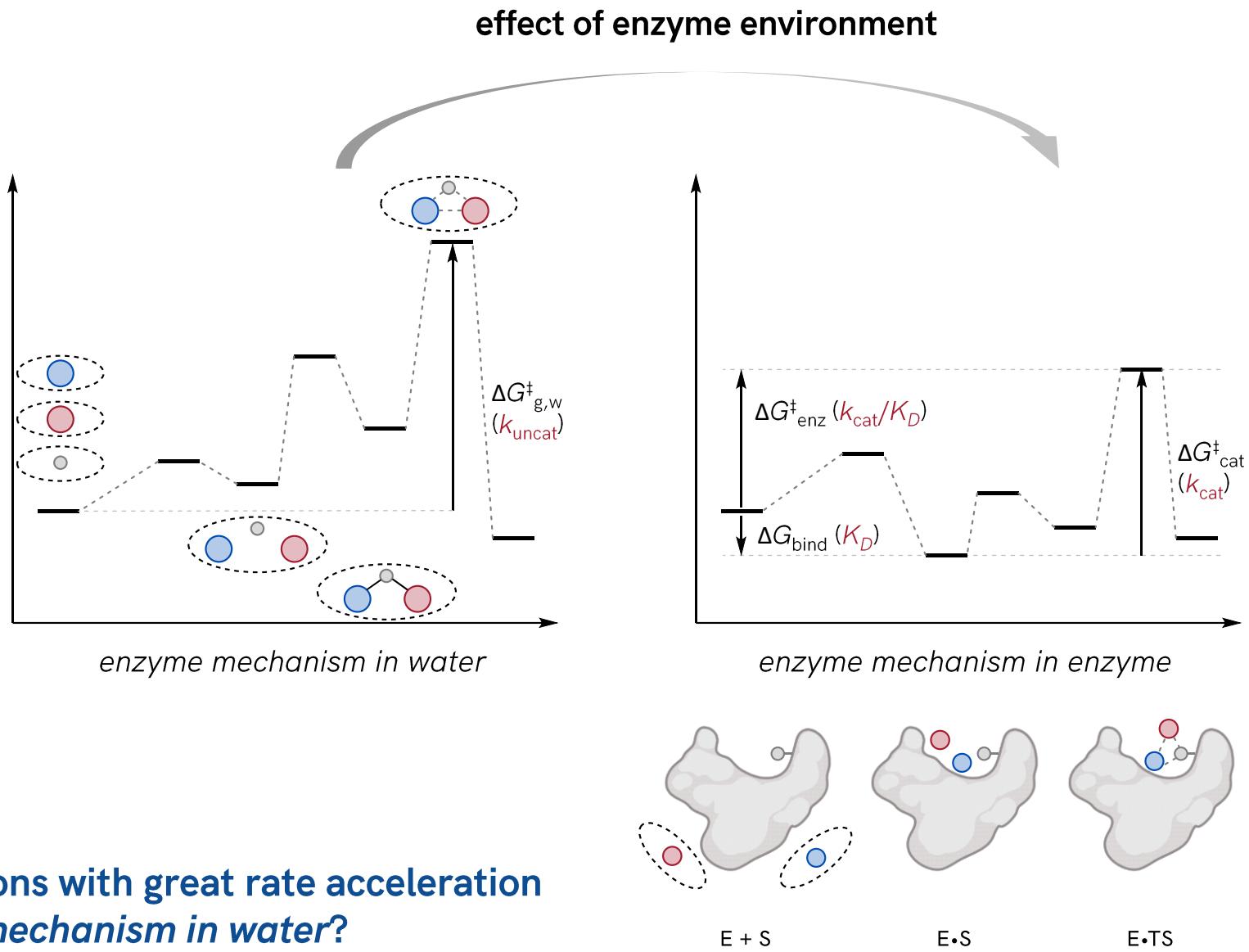


The reference reaction in water has to follow the same mechanism

The key energetic comparison is

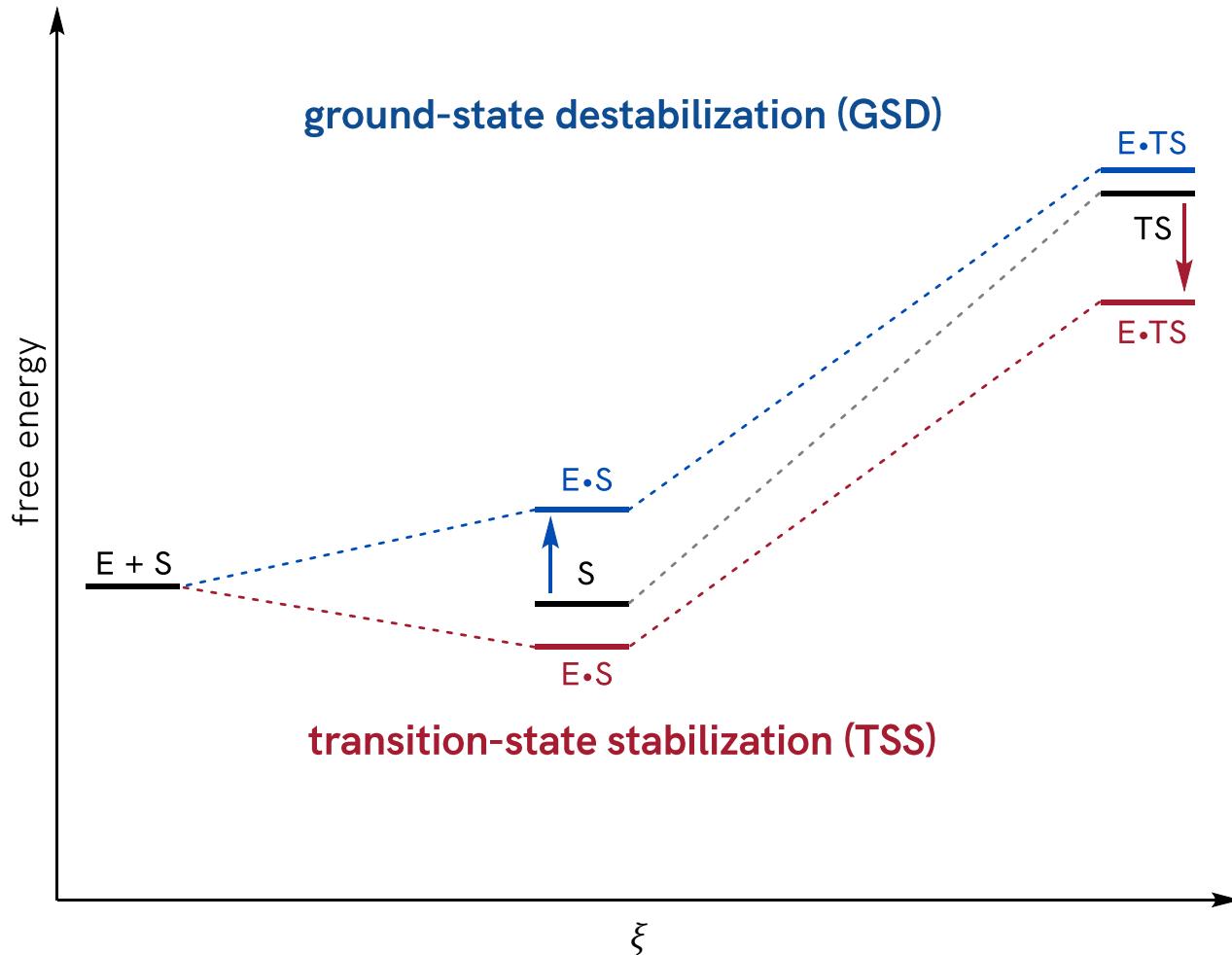
$$(\Delta G_{p,w}^{\ddagger} - \Delta G_{cat}^{\ddagger}) \propto \frac{k_{cat}}{k_{uncat}}$$

system ^b	$\Delta g_{w,w}^{\ddagger}$	$\Delta g_{p,w}^{\ddagger}$	$\Delta g_{cat}^{\ddagger}$
KI	27.0	22.1	11.2
AR	22.5	22.5	14.8
CA	23.8	23.8	11.0
CM	24.5	24.5	15.4
trypsin	32.0	26.0	18.0
DhlA	27.0	27.0	15.3
AP	27.5	27.5	15.2
Ras/G	27.5	27.5	16.1
TIM	26.4	28.4	14.0
Ach	36.0	29.5	13.5
lysozyme	33.6	31.5	18.0
Rb (MI)	32.0	32.0	15.0
Rb (DI)	47.0	36.0	15.0
ATPase	37.0	37.0	14.8
Pol T7	32.0	38.2	15.0
ODCase	38.8	40.0	15.4
Kf	36.0	46.4	19.0
SNase	36.0	51.5	14.9



How do enzymes promote reactions with great rate acceleration relative to the same mechanism in water?

Ground-state destabilization (GSD) and transition-state stabilization (TSS) are key



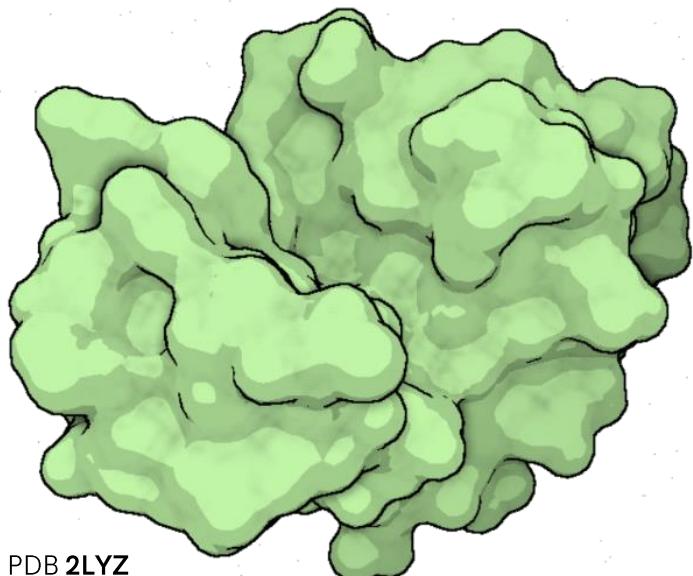
involves an active region of the surface of the enzyme which is closely complementary in structure not to the substrate molecule itself, in its normal configuration, but rather to the substrate molecule in a strained configuration, corresponding to the “activated complex” for the reaction catalyzed by the enzyme; the substrate molecule is attracted to the enzyme, and caused by the forces of attraction to assume the strained state which favors the chemical reaction

Pauling C&EN 1946

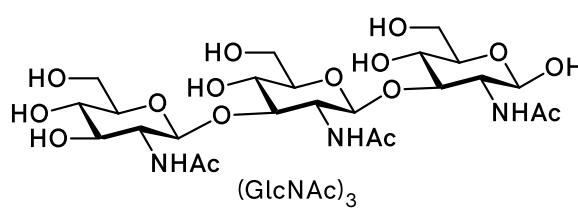
is responsible for all biological specificity. I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyse, that is, to the molecular configuration that is intermediate between the reacting substances and the products of reaction for these catalysed processes. The attraction of the enzyme molecule for the activated complex would thus lead to a decrease in its energy, and hence to a decrease in the energy of activation of the reaction, and to an increase in the rate of the reaction. Although

Pauling Nature 1948

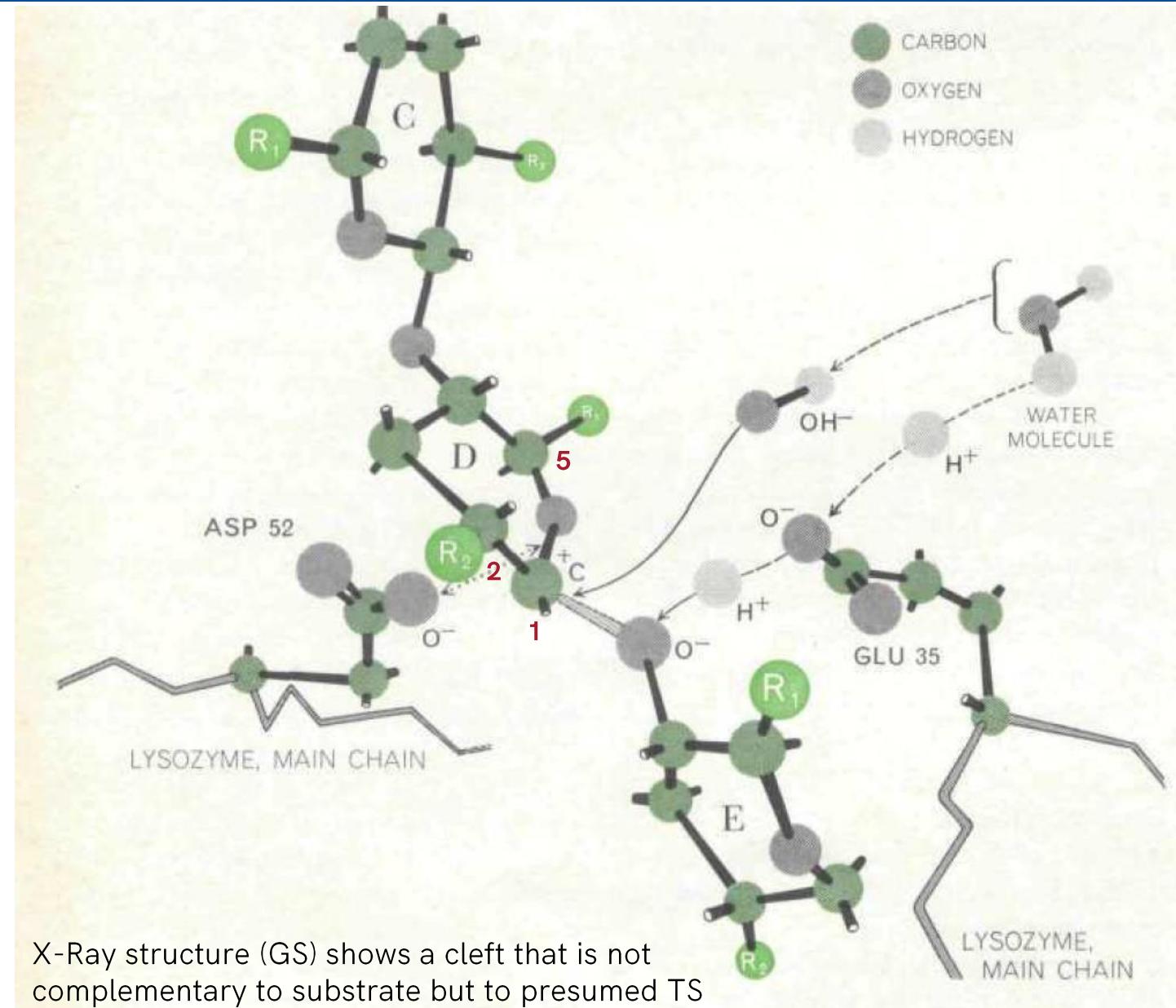
GSD was favored based on first XRD lysozyme structure



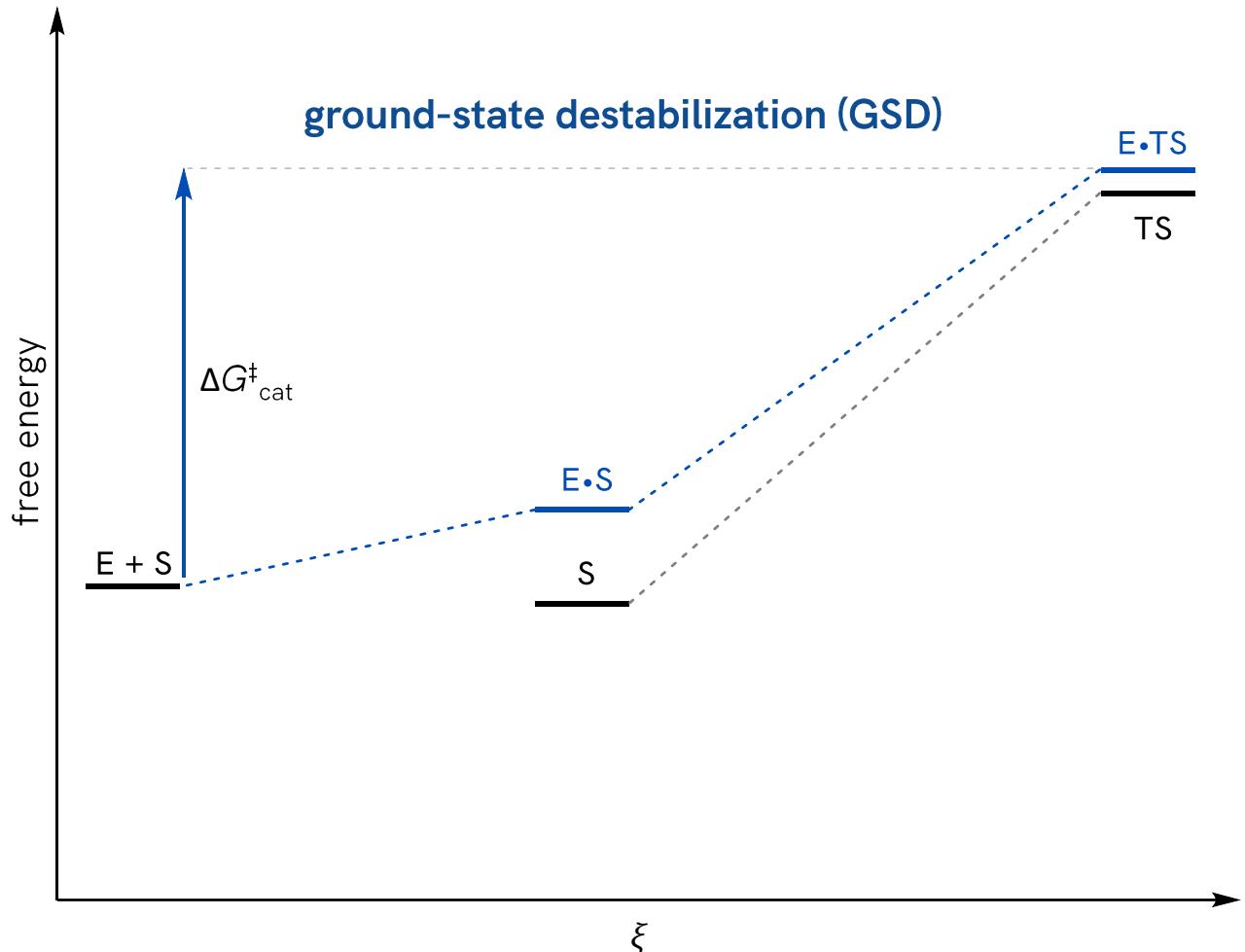
PDB 2LYZ



"CHAIR" CONFIGURATION (gray) is that normally assumed by the rings of amino sugar in the polysaccharide substrate. When bound against the lysozyme, however, the D ring is distorted (color) so that carbon atoms 1, 2 and 5 and oxygen atom 5 lie in a plane. The distortion evidently assists in breaking the substrate below the D ring.



GSD is not thermodynamically feasible as-is



involves an active region of the surface of the enzyme which is closely complementary in structure not to the substrate molecule itself, in its normal configuration, but rather to the substrate molecule in a strained configuration, corresponding to the “activated complex” for the reaction catalyzed by the enzyme; the substrate molecule is attracted to the enzyme, and caused by the forces of attraction to assume the strained state which favors the chemical reaction

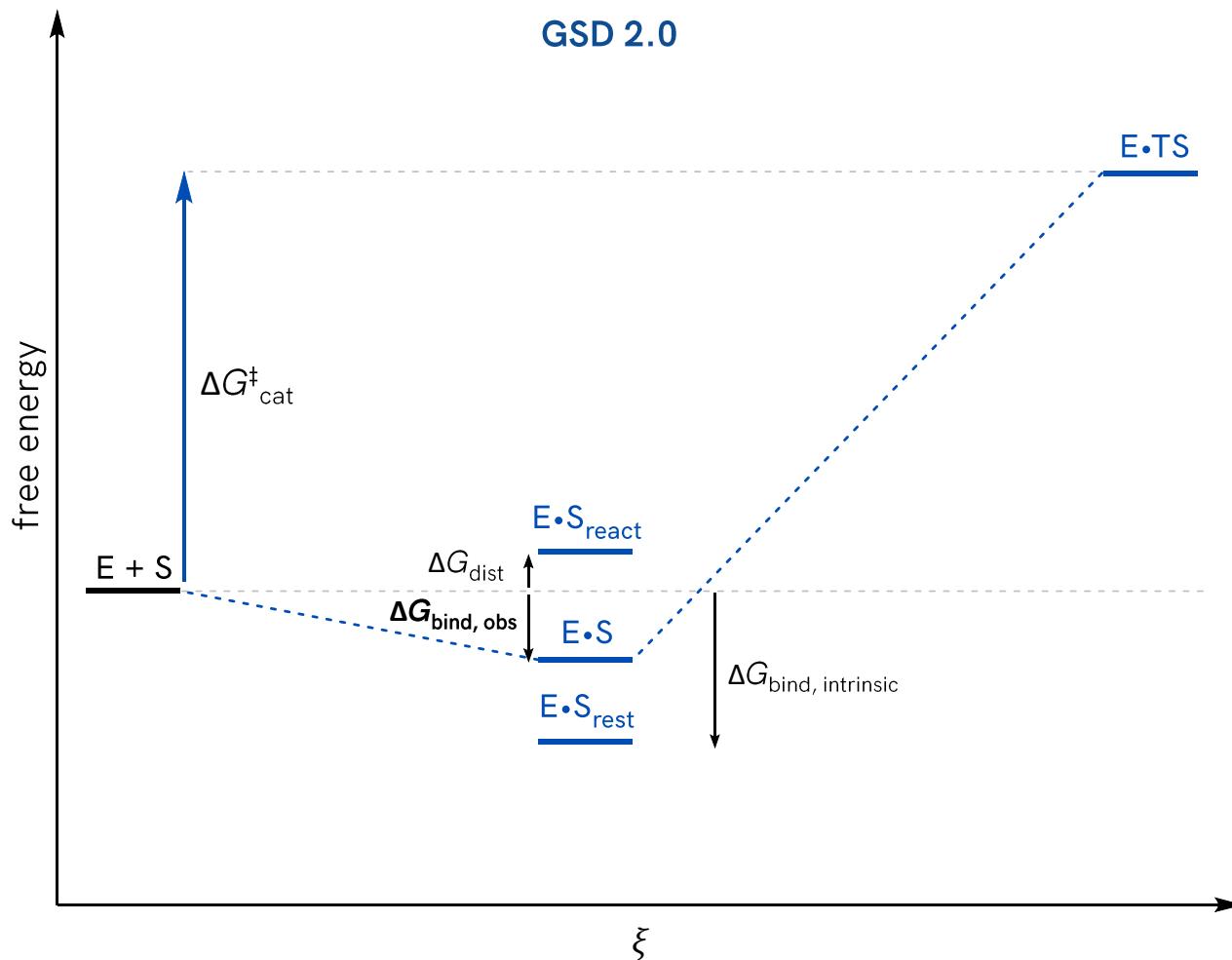
Pauling C&EN 1946

Issues

1. ground state is still $E + S$ so the barrier height is not reduced
2. enzymes are not stable enough (too soft) to distort covalent bonds

therefore substrate binding must still be thermodynamically downhill

The Circe effect: enzyme binds non-reactive region and destabilizes reactive region

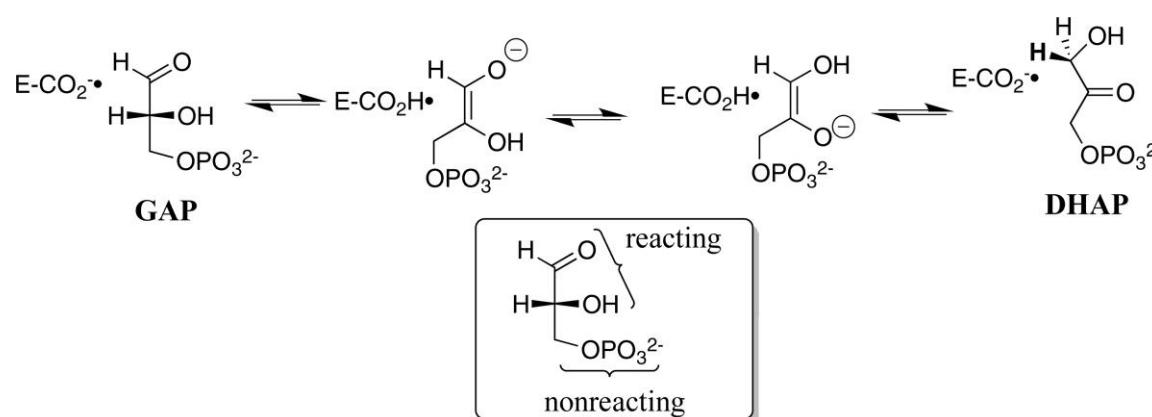


We will consider here the proposition that the *intrinsic binding energy* that results from the noncovalent interaction of a specific substrate with the active site of the enzyme is considerably larger than is generally believed. An important part of this binding energy may be *utilized* to provide the driving force for catalysis, so that the observed binding energy represents only what is left over after this utilization. Thus, the addition of a substituent that provides additional specific binding energy may result in little or no increase, or even in a decrease, in the observed binding if binding energy is used up to increase the reaction rate by causing a localized destabilization, conformation change, more productive binding, or greater loss of entropy in the enzyme–substrate complex.

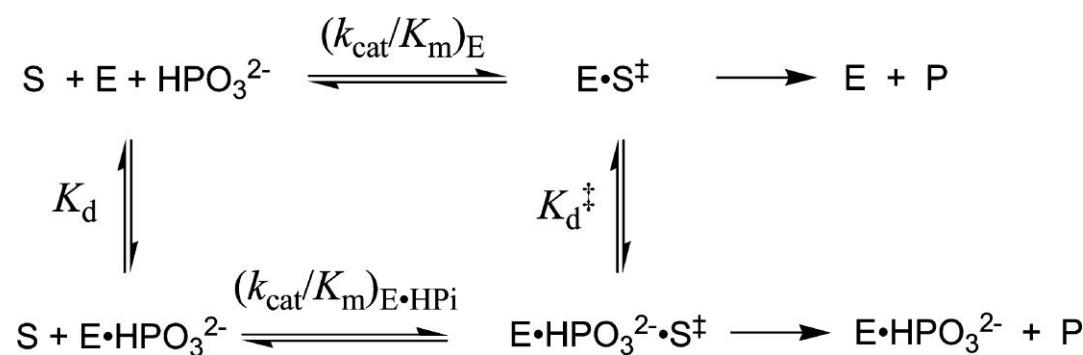
Jencks 1975

Truncated substrates react much more slowly but can be rescued by fragments

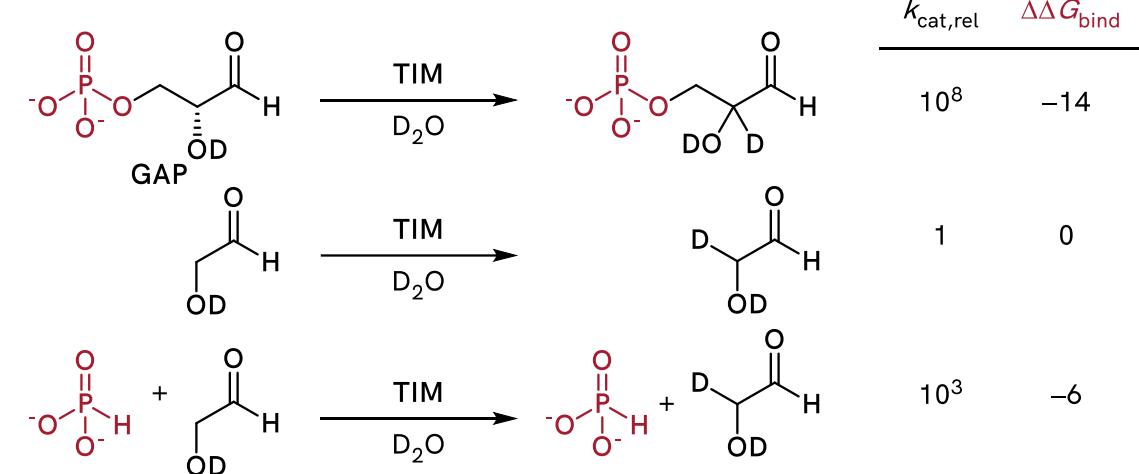
Triosephosphate isomerase (TIM) catalyzes **GAP** to **DHAP**



The affinity of phosphate to enzyme is K_d in the GS and K_d^\ddagger in the TS.



Truncated substrate reacts slowly but is rescued by phosphite



The “Circe effect”

$\Delta G_{bind,phosphite}$ pays for conformational change from inactive (open) to active (closed) form of TIM that is optimal for TS stabilization

Accounting for the remaining $14 - 6 = 8$ kcal/mol:

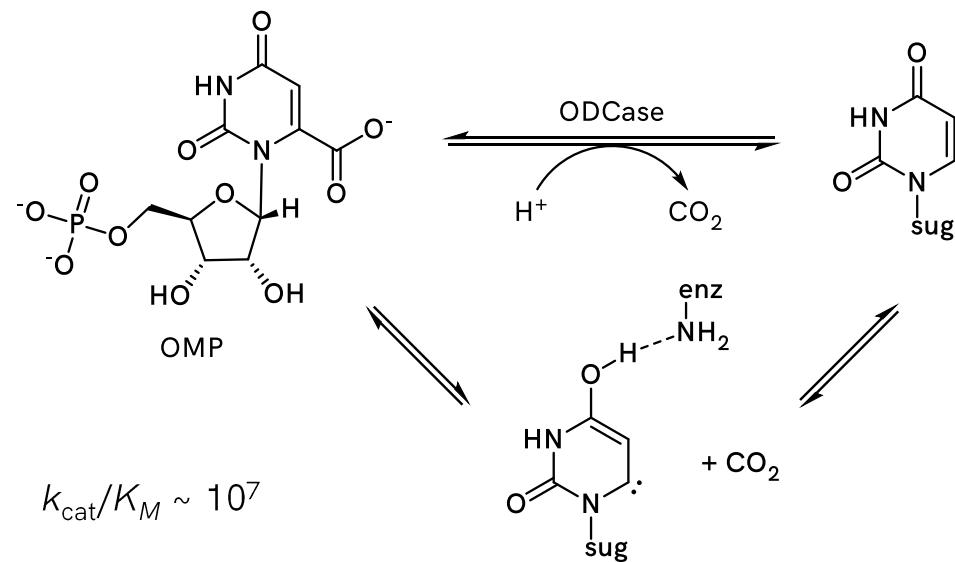
1. entropic price of binding extra component
 2. imperfect fit of fragmented TS rel. **GAP** TS
 3. bridging O in **GAP** is involved in key NCIs to active site

From the binding constants we can obtain the intrinsic $\Delta G_{bind, phosphite}$

Desolvation leads to GSD of reacting fragments

Desolvation

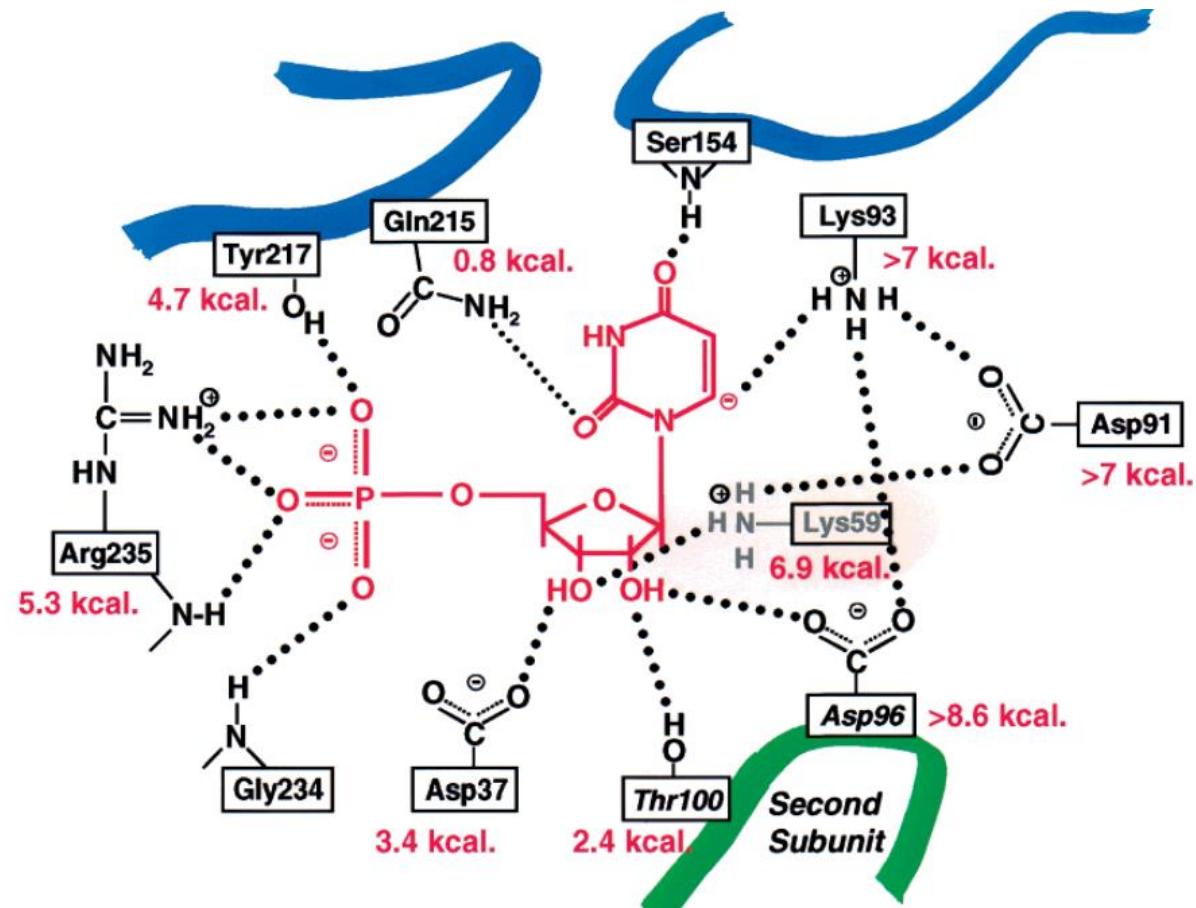
Charged GS is disfavored in low-dielectric active site relative to water, leading to destabilization



Computations suggest that low-dielectric environment is necessary for rate acceleration

Reaction	$\Delta H^\ddagger (\text{kcal mol}^{-1})$			
	($\epsilon = 1$)	($\epsilon = 4$)	($\epsilon = 10$)	($\epsilon = 78.54$)
Uncatalyzed	+43.9	+41.5	+42.5	+42.4
Catalyzed	-61.9	+17.6	+33.7	+43.2
Uncatalyzed - catalyzed	+105.8	+23.9	+8.8	-0.8

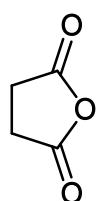
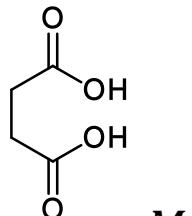
Later XRD structure shows that active site is in fact polar, and Asp96 mutation leads to weaker binding:



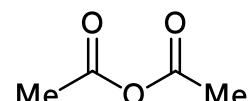
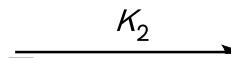
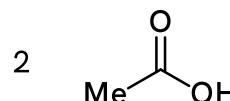
Enzymes use binding energies to pay for unfavorable but reactive conformations

Entropy trap

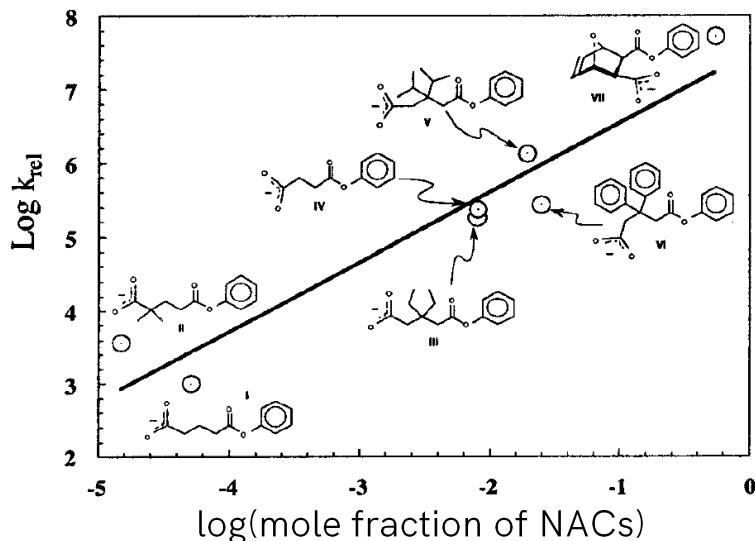
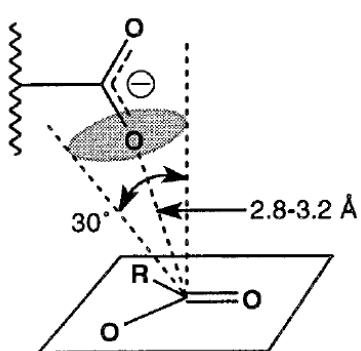
bringing reactive groups closer leads to high effective molarity



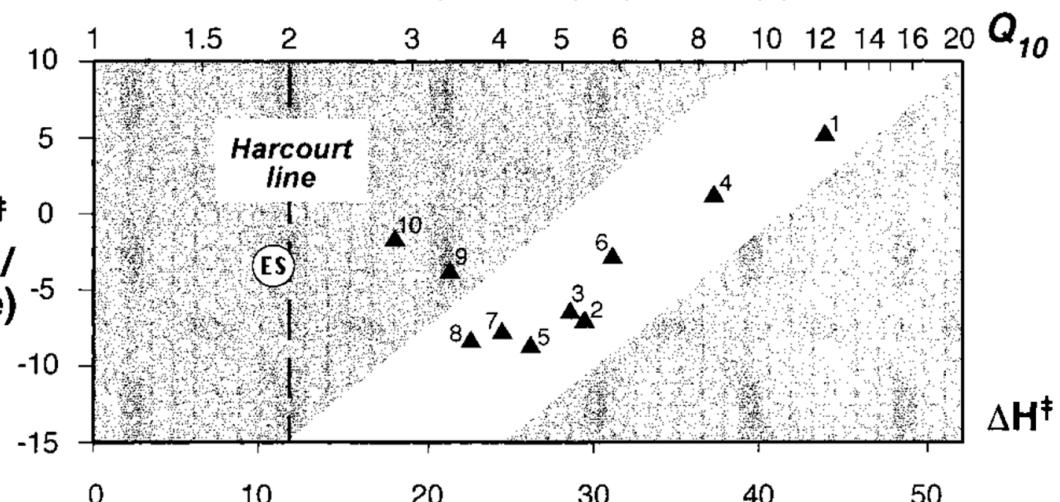
$$M_{eff} = \frac{K_1}{K_2} = 3 \times 10^5$$



"Near attack conformers": GS conformers resembling the TS



But enzyme-catalyzed reactions are known to be temperature-dependent and therefore driven primarily by enthalpy!



Near-attack conformers

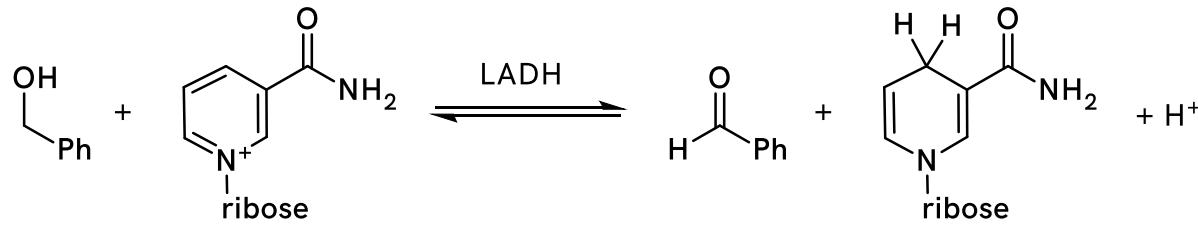
Enzymes bind NACs almost as tightly as TSs, so preferential TS binding is not requisite for catalytic activity

Corollaries:

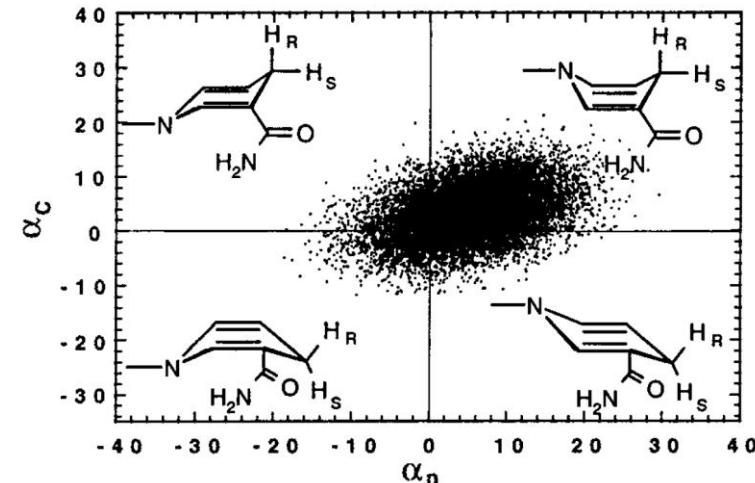
1. Enzyme does not undergo significant changes in converting from E.NAC to E.TS
2. Unlike in solution, active site polar groups do not significantly reorganize when entering TS. This energy saving leads to catalysis
3. Dipoles are fixed when NAC is formed

Liver alcohol dehydrogenase biases the conformer distribution of DHP

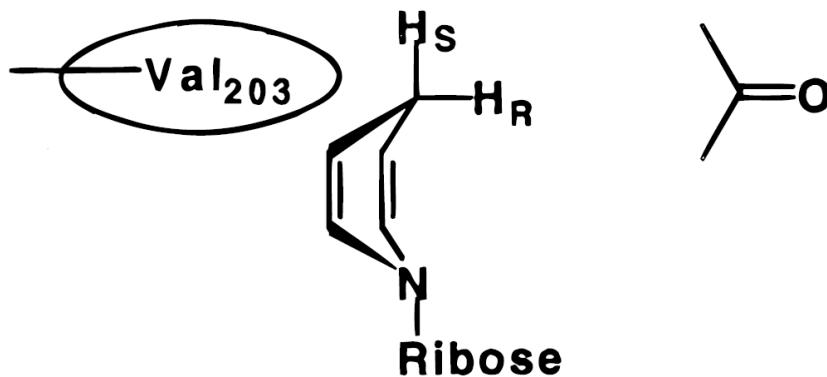
LADH catalyzes the interconversion of alcohols and aldehydes



MD simulations show bias towards quasi-boat conformer



The key Val203 residue drives anisotropic motion toward the substrate



Catalytic efficiency drops as a function of steric bulk at Val position:

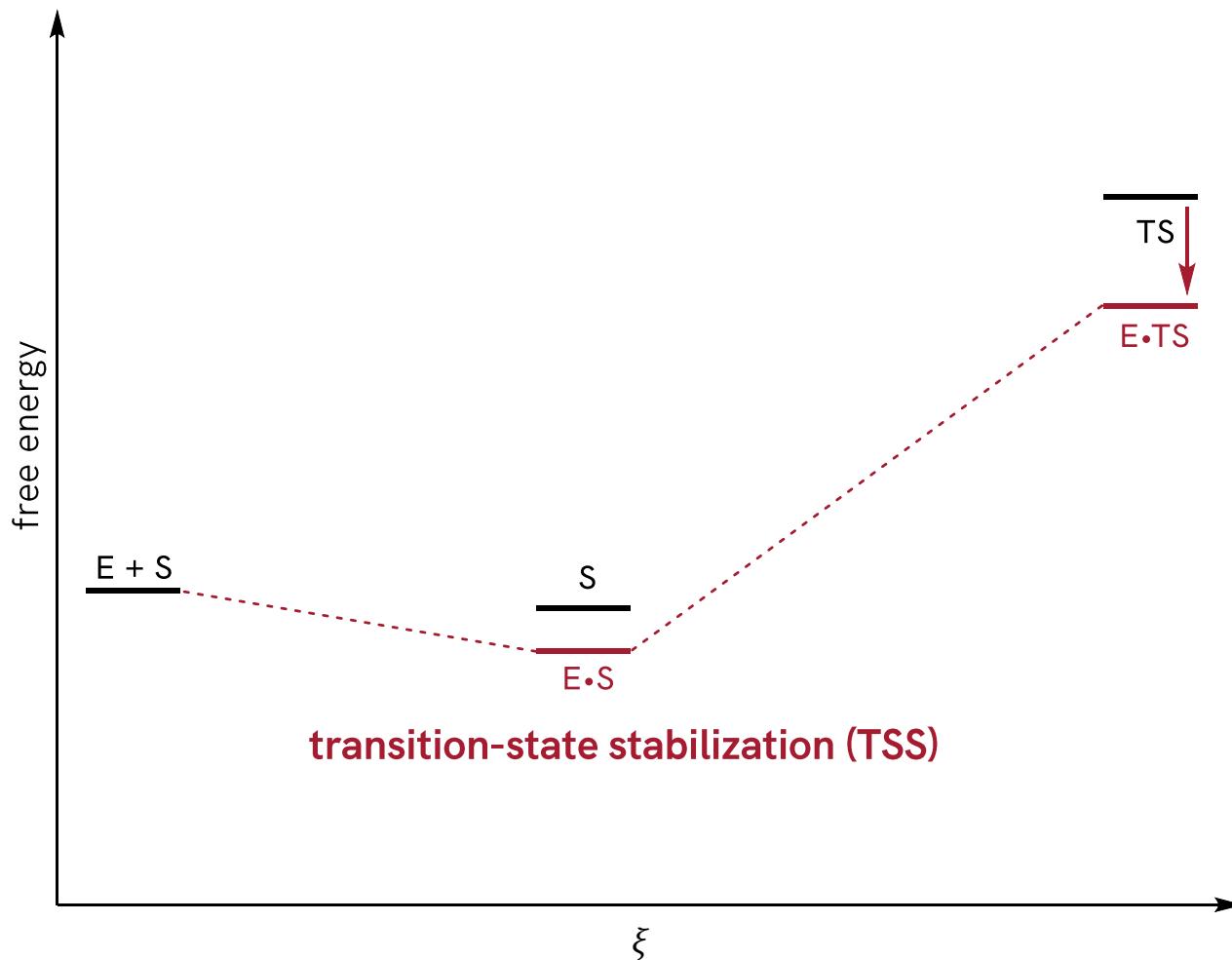
V203G leads to 100-fold lower catalytic efficiency
i.e. bulky side chain promotes NAC formation

Caveats:

1. The effect of mutations on catalysis (10^2) is small.
2. By the NAC definition, the same effects that stabilize the NAC will stabilize the TS, so this is not different from TSS.

This explanation (alongside GSD and TSS) is just a description of a phenomenon; it does not explain how enzymes achieve catalysis!

Transition-state stabilization (TSS) seems intuitive, but is it?



is responsible for all biological specificity. I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyse, that is, to the molecular configuration that is intermediate between the reacting substances and the products of reaction for these catalysed processes. The attraction of the enzyme molecule for the activated complex would thus lead to a decrease in its energy, and hence to a decrease in the energy of activation of the reaction, and to an increase in the rate of the reaction. Although

Pauling *Nature* 1948

Issues

1. catalytic antibodies raised against TS analogs have incredible affinity for them but much poorer rate acceleration
2. no small-molecule “enzyme mimic” can exhibit enzyme-level catalytic efficiencies

How do enzymes achieve stabilization of the TS relative to the same TS in water?

TSS is proposed to be driven largely by electrostatic effects

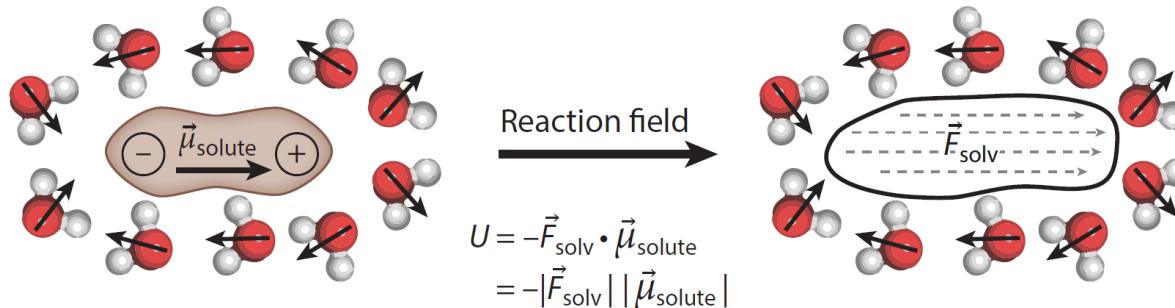
Electrostatic preorganization/Electric field catalysis

Enzyme active sites provide a preorganized polar environment that stabilizes the TS more than the GS, or:
Enzyme active sites feature electric fields that stabilize the dipole moment of the TS more than the GS

Electrostatics involves charges, dipoles, polarizability (i.e. most NCl's); does not involve vdW strain, entropy, and dynamical effects.

Electric fields in simple solution

All electrostatic contributions can be unified into an electric field:

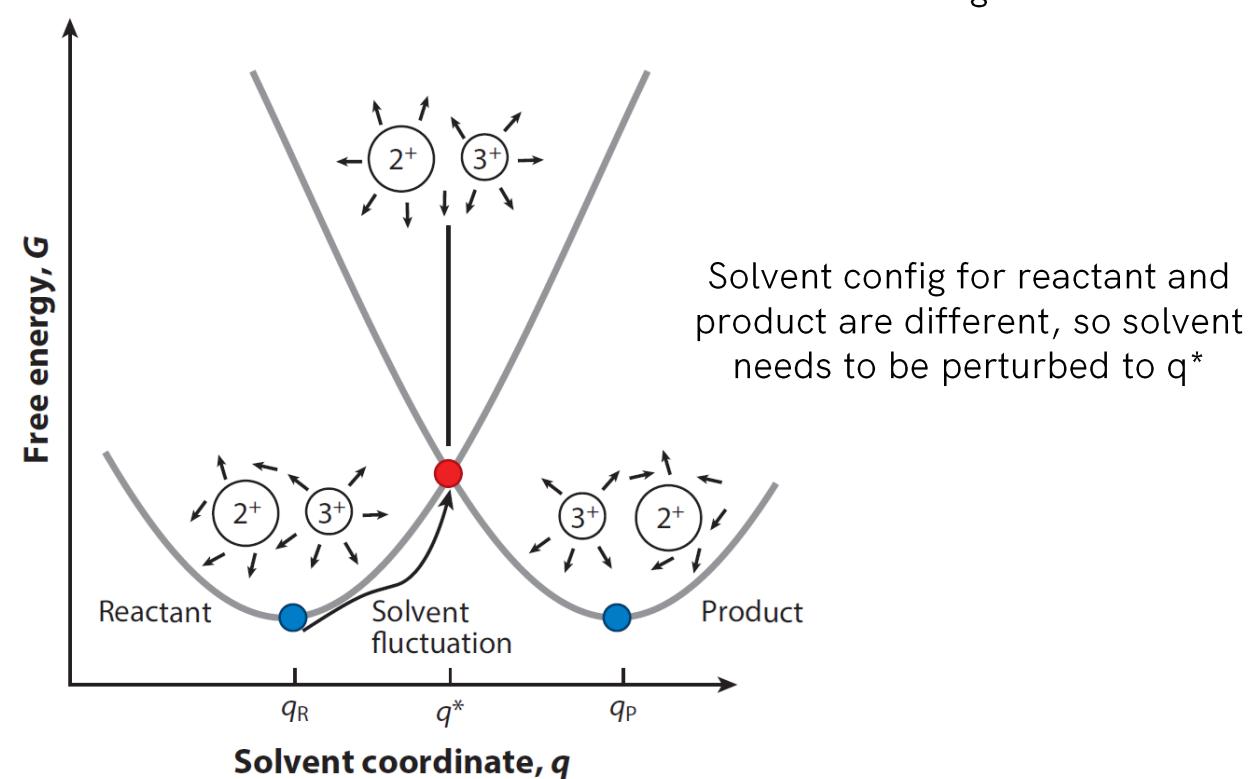


Solute dipole forces solvent molecules to align

Align solvent molecules create a nonzero field (the "reaction field")

Interaction between dipole and field leads to solvation energy U

Consider a thermoneutral $\text{Fe}^{2+} - \text{Fe}^{3+}$ exchange:



q^* solvates reactant state much more poorly than q_R , so it is rarely sampled, but once it is sampled, the electron exchange happens

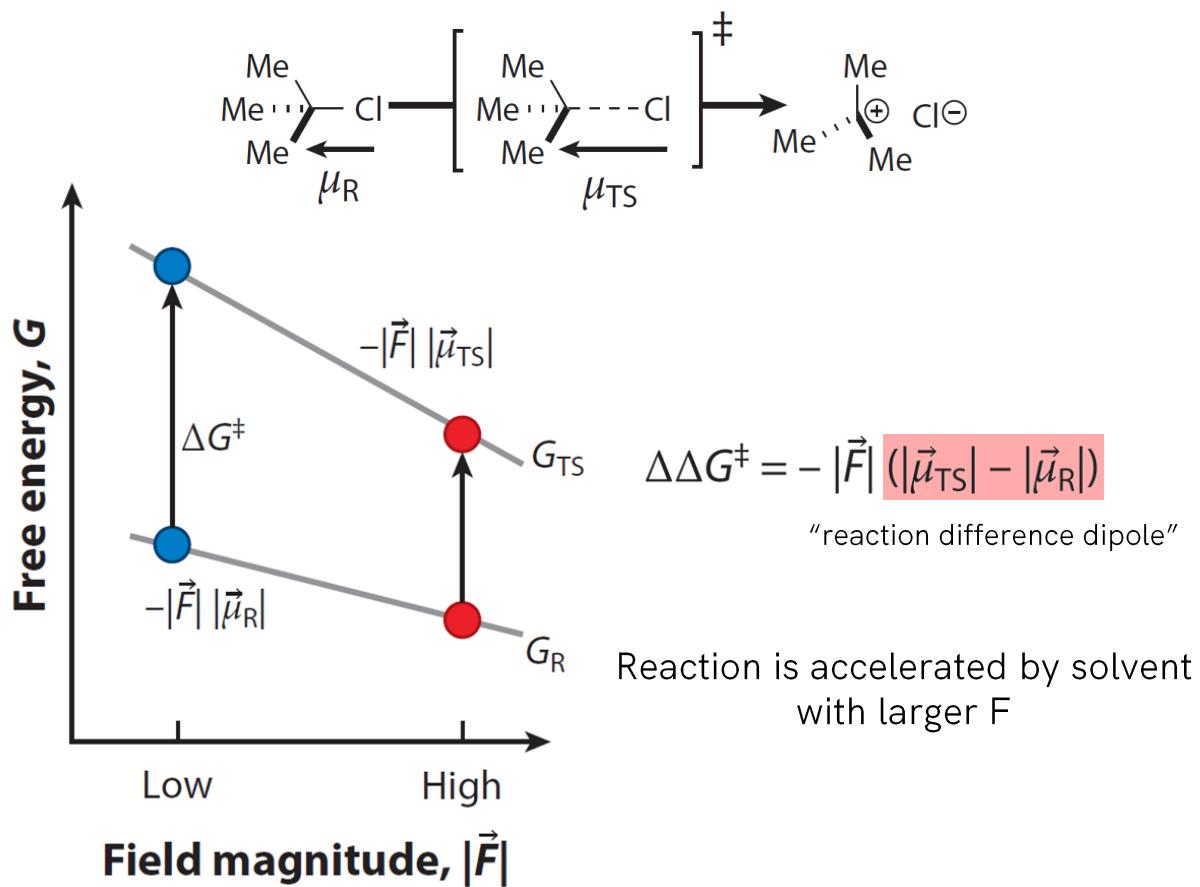
i.e. solvent reorganization is the rate-limiting step here

Electric field catalysis can be described by dipole–field interactions in the GS and TS

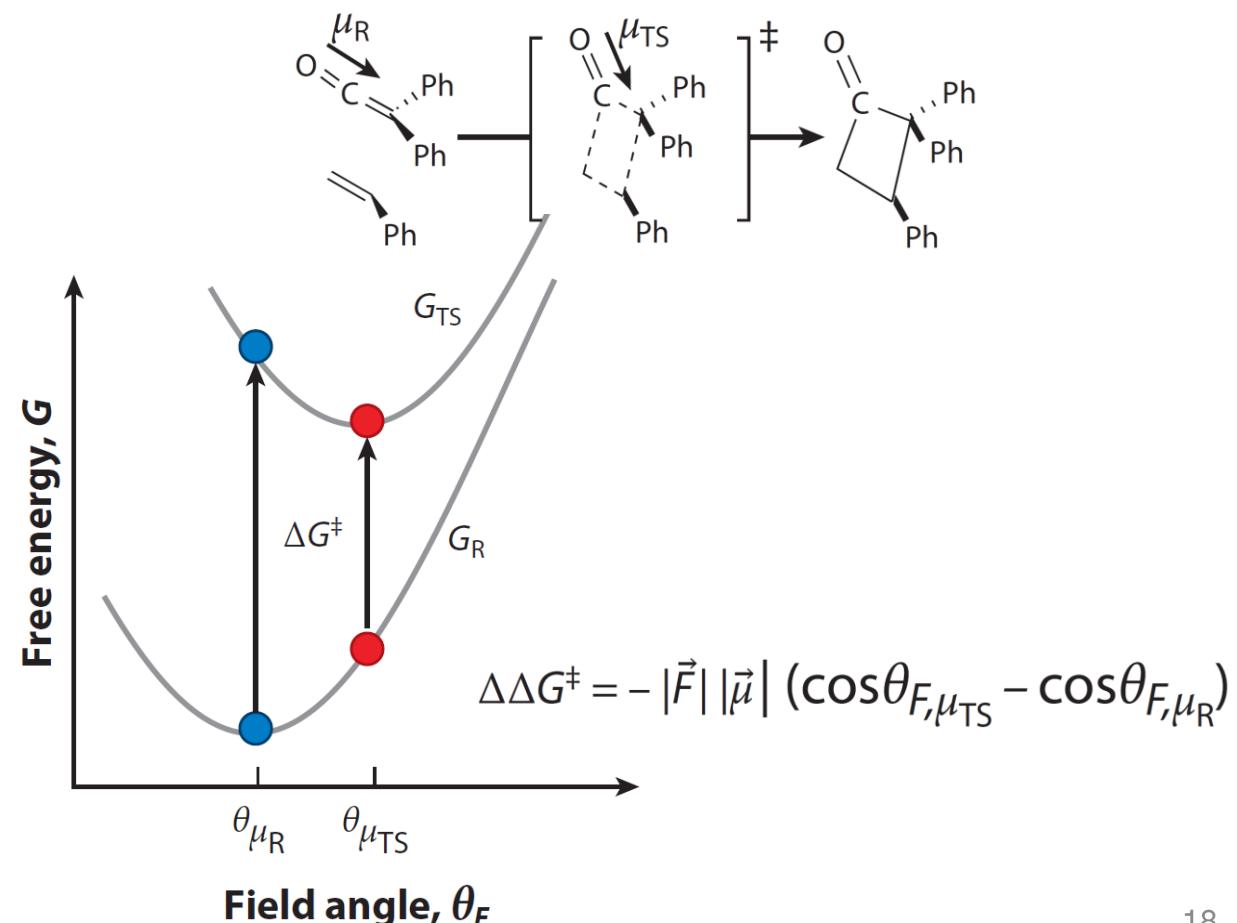
$$\Delta\Delta G^\ddagger = - \left((\vec{F}_{\text{env,TS}} \cdot \vec{\mu}_{\text{TS}}) - (\vec{F}_{\text{env,R}} \cdot \vec{\mu}_{\text{R}}) \right)$$

Dipole changes from GS to TS, so an environment that stabilizes the TS dipole more than the GS dipole would catalyze the reaction.

Case 1: dipole changes in magnitude



Case 2: dipole changes in direction



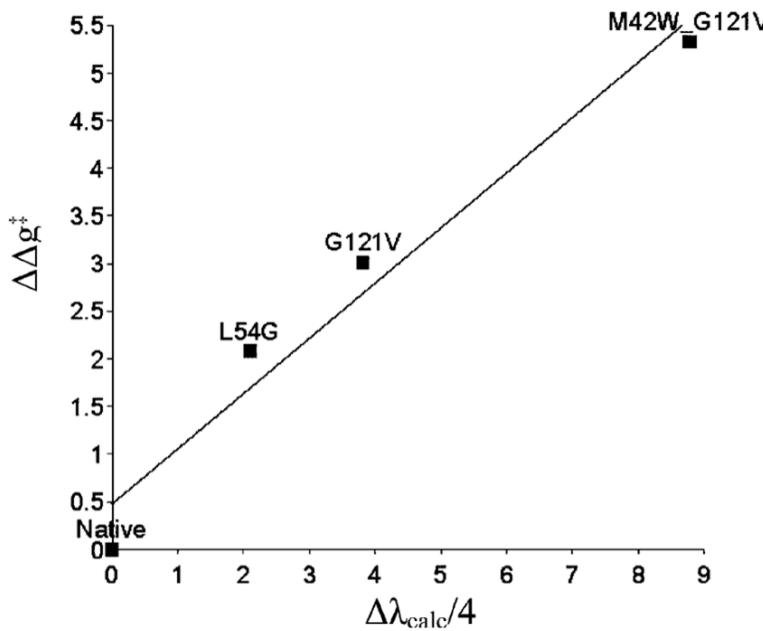
A plethora of computational studies establish that electrostatics is the dominant driver of catalysis

Electrostatics are a major TSS driver:

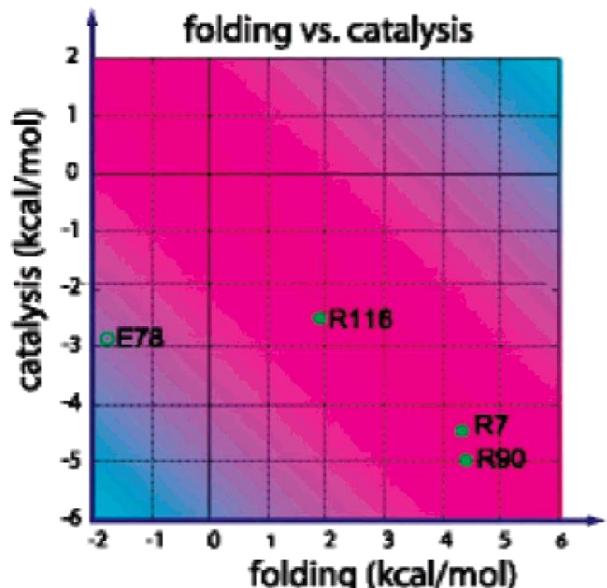
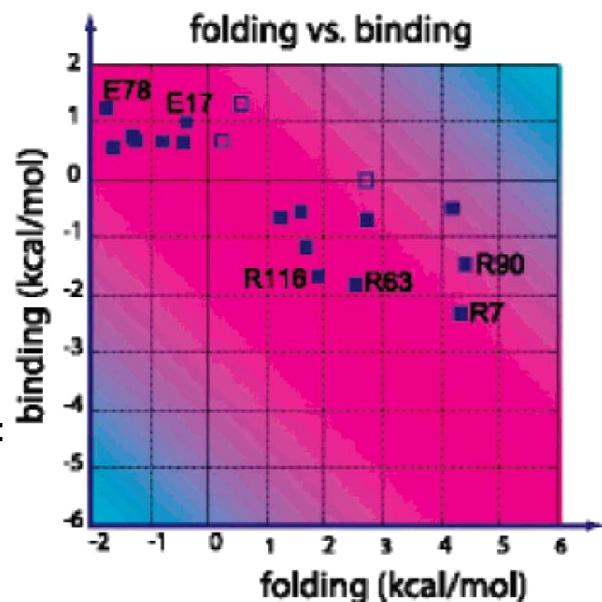
Table 3. Electrostatic Contribution to the Catalytic Effects of Specific Enzymes^a

system	$(\Delta\Delta g_{\text{tot}}^{\ddagger})_{\text{calc}}$	$(\Delta\Delta g_{\text{elect}}^{\ddagger})_{\text{calc}}$	$(\Delta\Delta g_{\text{cage}}^{\ddagger})_{\text{calc}}$	$\Delta\Delta g_{\text{obs}}^{\ddagger}$	ref
DhlA	11.6	8 ^b (8)	2.3	11.7	95, 96
CM	10.3	8 (14)	0.0	9.1	90
ODCase	19.0	17 ^c		23.0	36
ribosome	8.0	– (8)	0.0	6.0	97

Mutations that increase reorganization of enzyme (λ) lead to lower rate:



Enzymes pay for preorganization using folding energy:



Stability of enzyme anticorrelates with binding/catalysis!

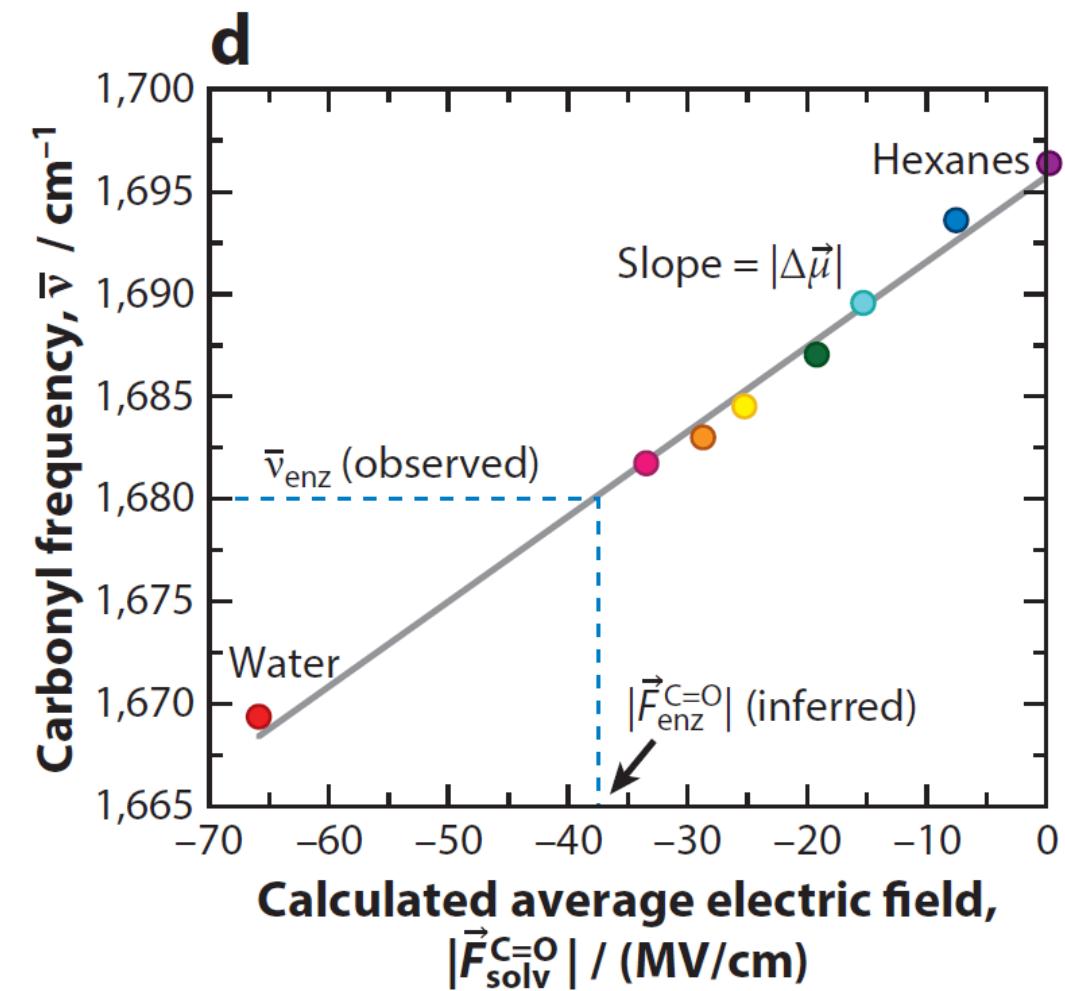
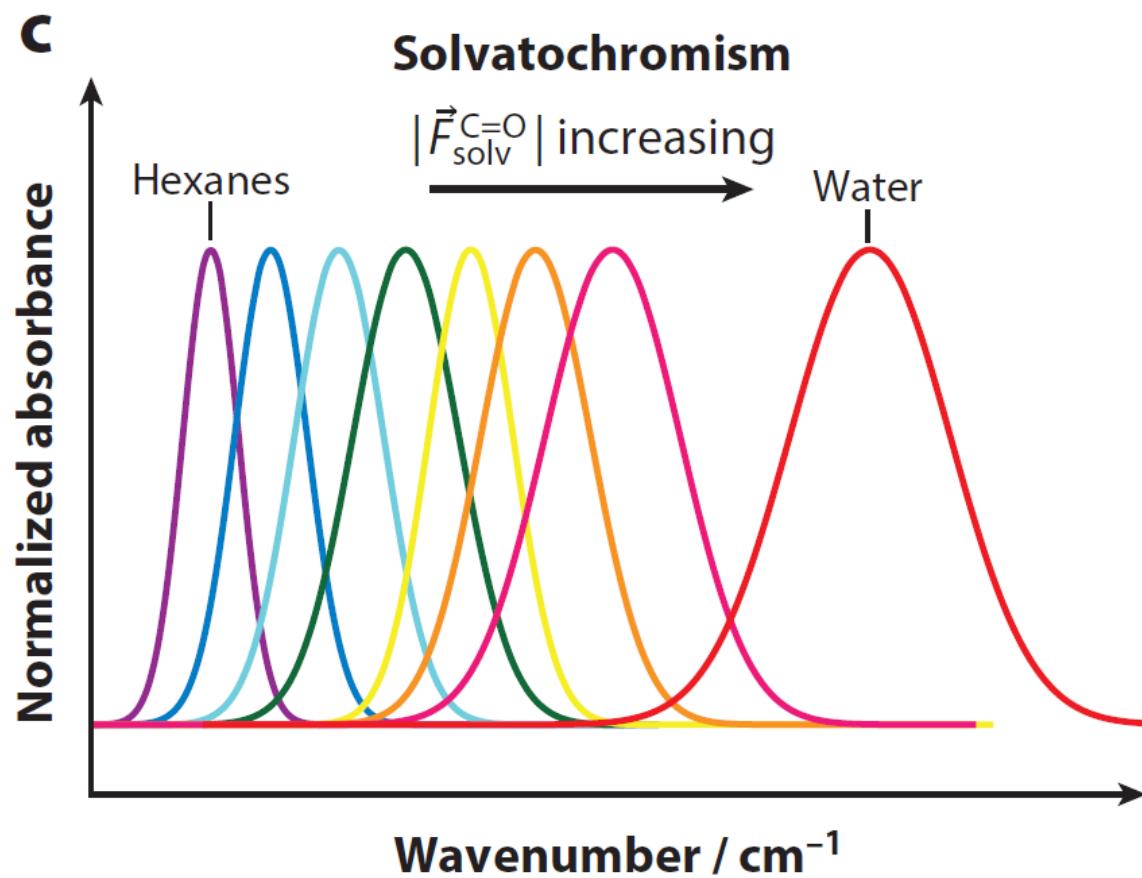
Despite these successes, these computational studies have not been fully accepted, primarily because electrostatic catalysis proved exceedingly difficult to quantify (or even identify) experimentally; this was not helped by repeated assertions that these effects can only be understood through computation (33). In contrast, we argue here that measurements of electric fields inside enzymes provide a path to understand and quantify electrostatic effects in catalysis.

Fried; Boxer *Annu. Rev. Biochem.* 2017

Vibrational Stark spectroscopy enables measurement of electric fields using probes

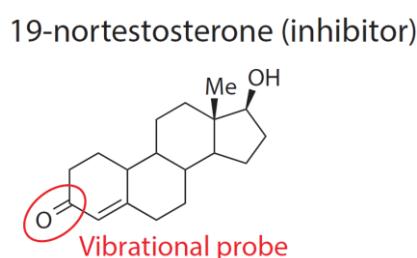
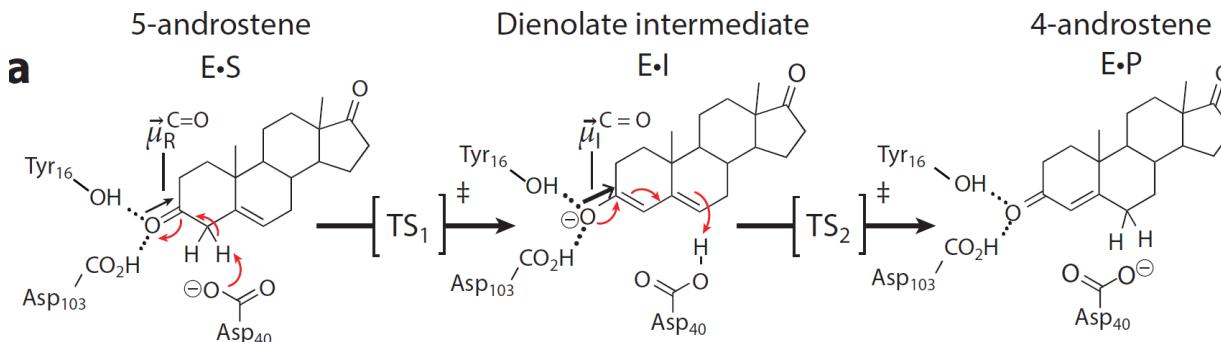
Electric fields affect the IR stretching frequency of C=O; different solvents have different (calculated frequencies).

Construct a calibration curve using measured frequency and calculated fields. Then measure the frequency in an unknown field.

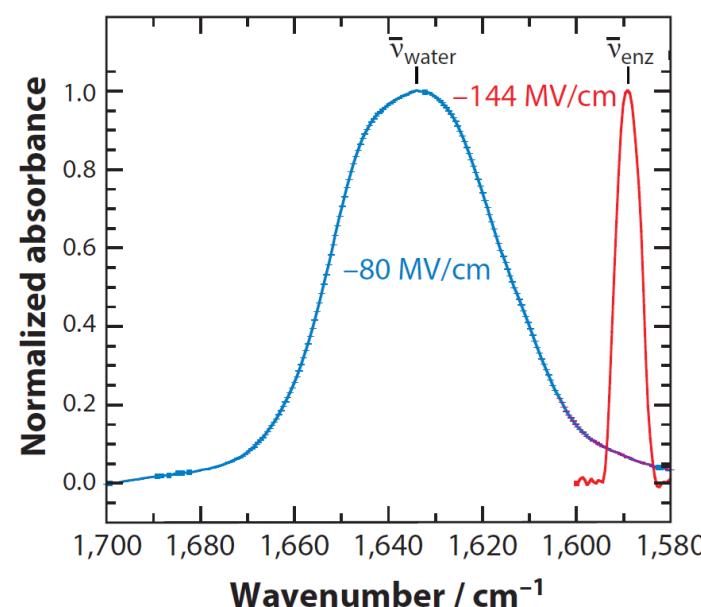


Ketosteroid isomerase needs to effect an unfavorable deprotonation

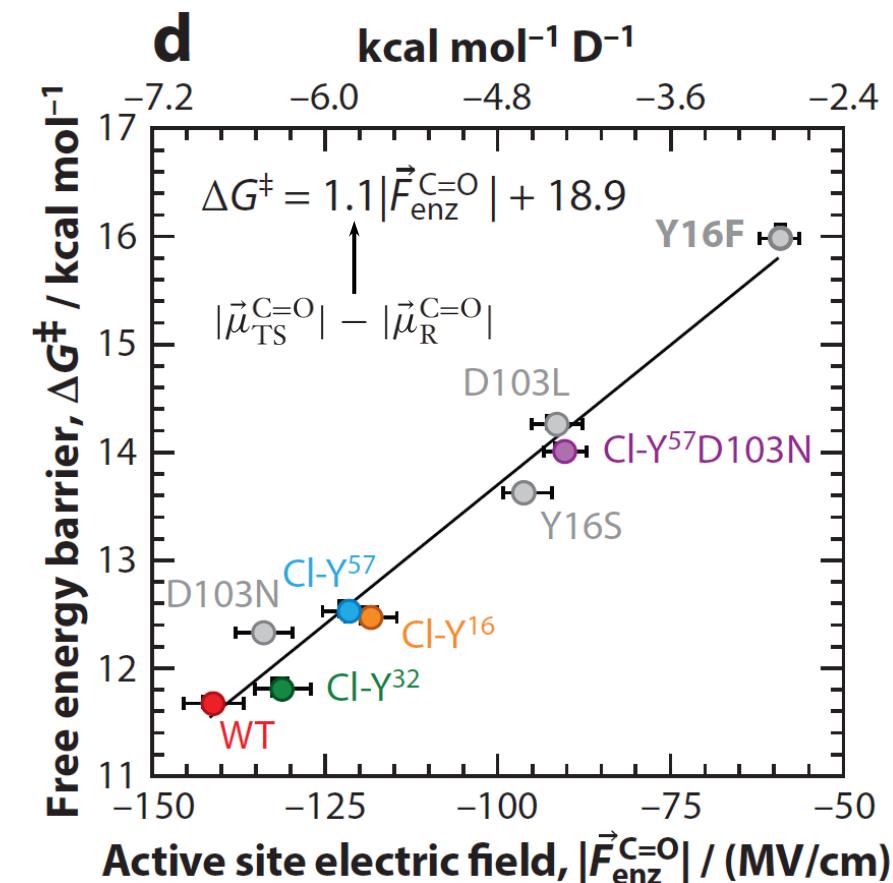
Case 1: dipole changes in magnitude



KSI exerts a large (redshift) and homogeneous (narrow) electric field on the inhibitor relative to water.



Mutations that impair catalysis decrease the electric field:



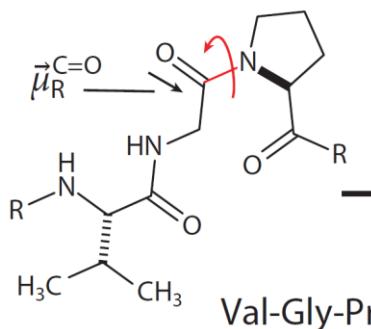
1.1 D is the “rate-limiting” increase in the dipole that the active site has to stabilize

the TS solvation energy contributes 72% of total rate acceleration.

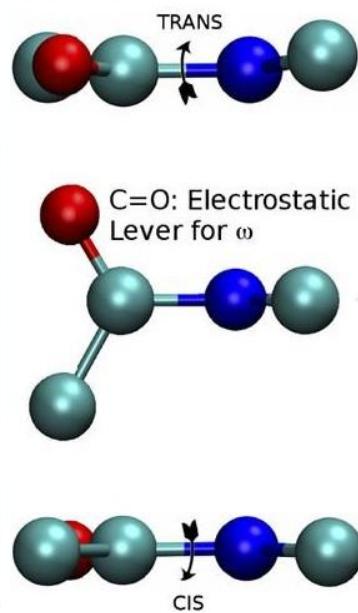
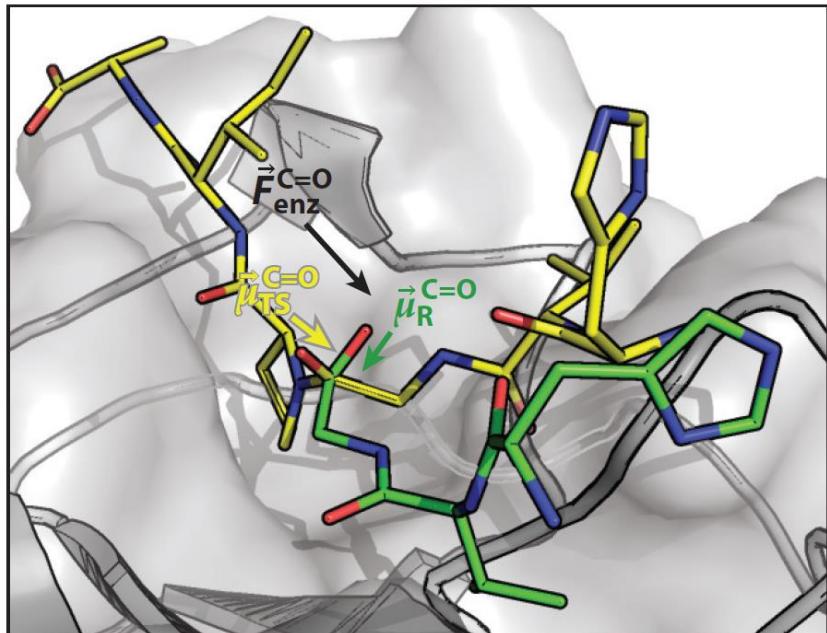
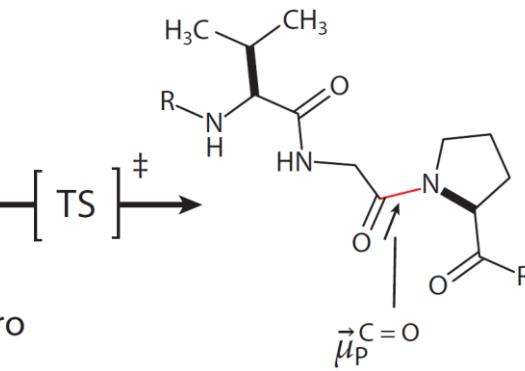
Cyclophilin A catalyzes a change in dipole orientation

Case 2: dipole changes in orientation

cis-Gly-Pro peptide



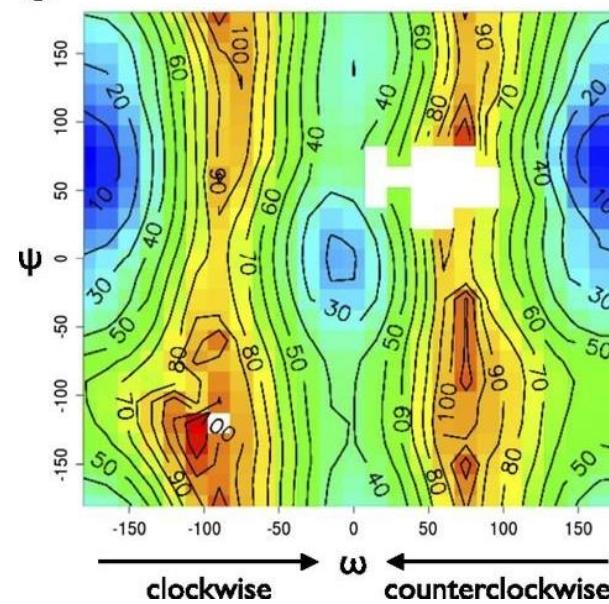
trans-Gly-Pro peptide



Electric field decreases barrier associated with CCW rotation by ~7 kcal mol⁻¹

C

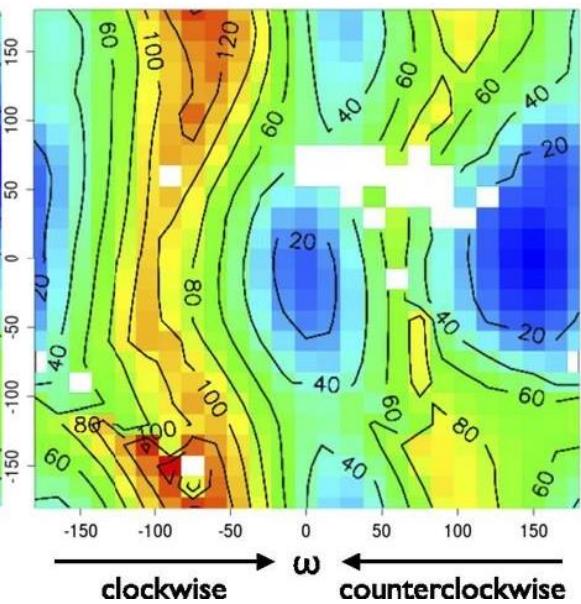
NO FIELD



clockwise

counter-clockwise

-Ez FIELD



clockwise

counter-clockwise

Enzyme employs electric field to twist dihedral
the TS solvation energy contributes 92% of total rate acceleration

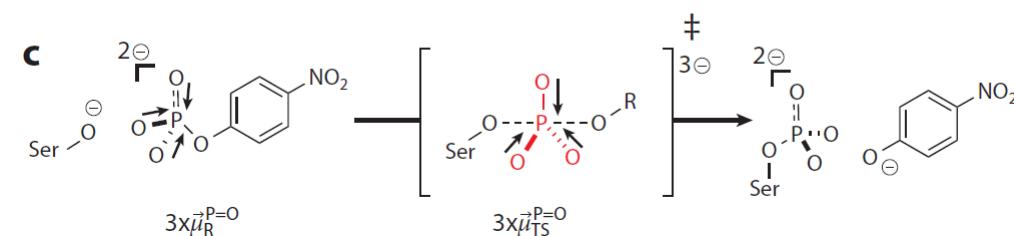
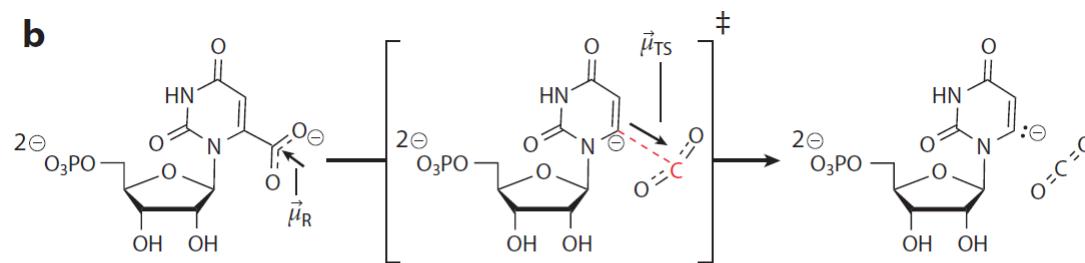
The field destabilizes the carbonyl locally, so distal interactions have to compensate, cf. the Circe effect.

Electric field catalysis is broadly consistent with enzyme behavior

Electrostatic preorganization/Electric field catalysis

Enzyme active sites provide a preorganized polar environment that stabilizes the TS more than the GS, or:
Enzyme active sites feature electric fields that stabilize the dipole moment of the GS more than the TS

1. Reactions with larger dipole reorientations are more catalyzable



Decarboxylases and phosphatases have large rate catalytic proficiencies

2. Reactions with small dipole reorientations are rare in nature

e.g. Diels-Alderases promote concerted pericyclic reactions with little charge rearrangement

3. Promiscuous enzymes recognize charge and not structure

e.g. alkaline phosphatase also has sulfatase activity;
based on point charge/dipole differences on S-O⁻ vs P-O⁻ we can completely account for difference in rate enhancement

Unanswered observations

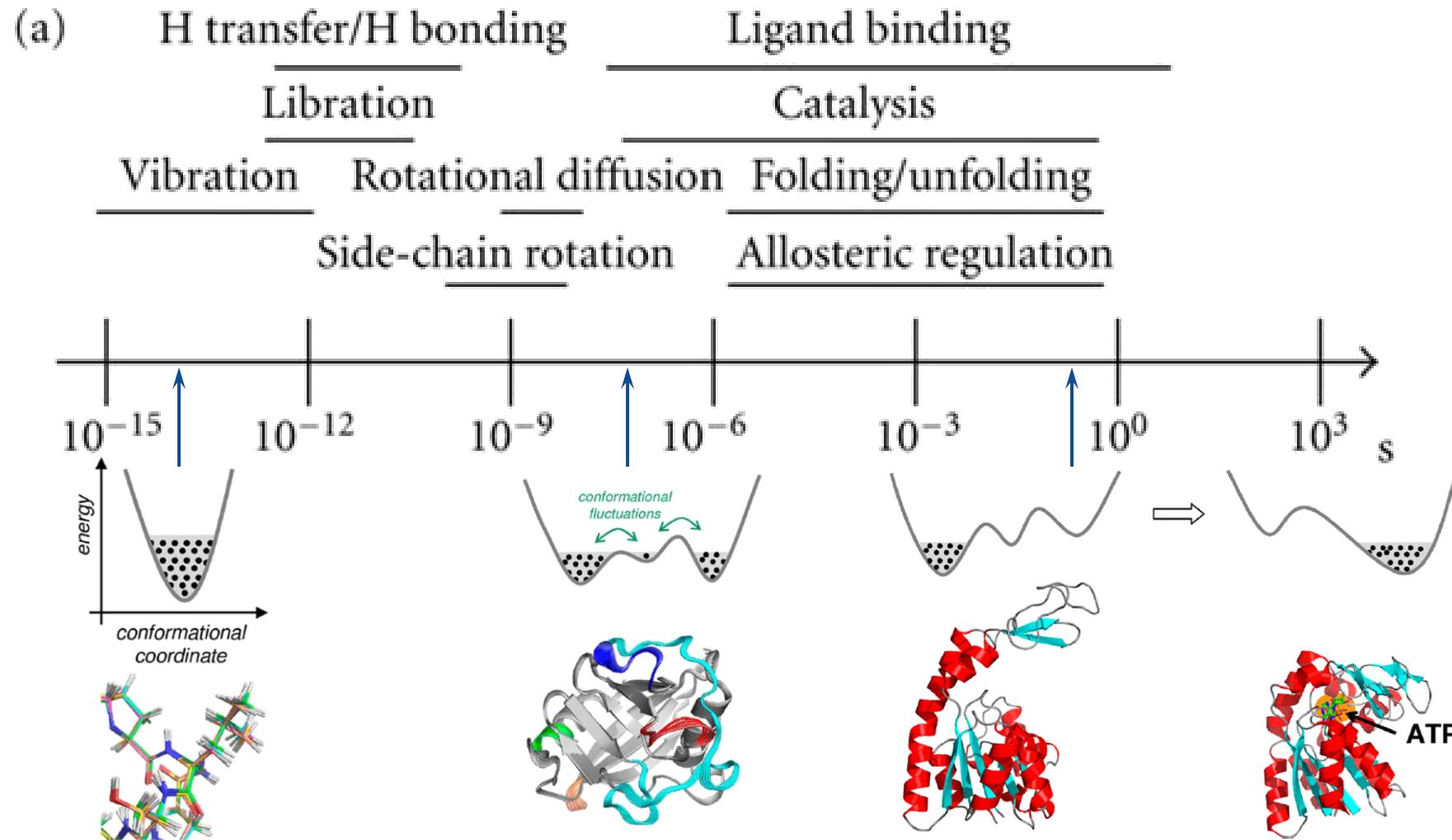
1. Distal mutations are often found to significantly affect catalysis
2. Virtually no small-molecule catalyst can achieve enzyme-levels of rate acceleration
3. Enzyme mechanisms involve multiple intermediates that cannot be accounted for by a single optimal active site organization

What physical processes lead to the formation of electrostatically preorganized active site for barrier crossing?

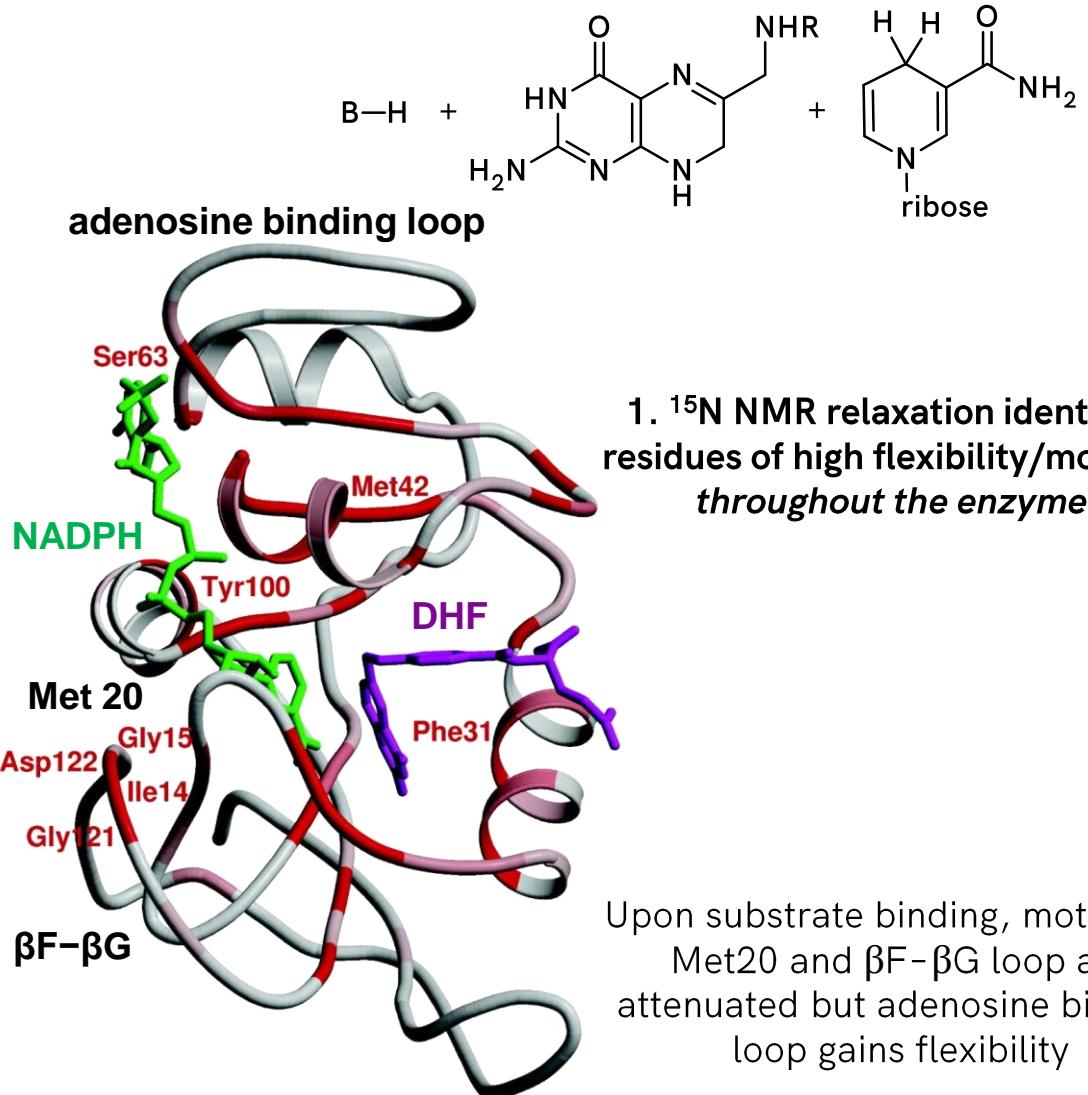
Enzyme dynamics is a leading hypothesis to explain catalysis

Enzyme dynamics

The coupled internal motions of an enzyme accelerate sampling of configurations that are conducive for barrier crossing



Early studies on dihydrofolate reductase identified a network of key residues

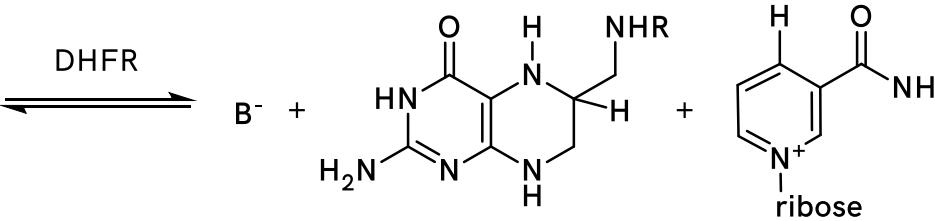


1. ^{15}N NMR relaxation identifies residues of high flexibility/mobility throughout the enzyme

Upon substrate binding, motions in Met20 and $\beta\text{F}-\beta\text{G}$ loop are attenuated but adenosine binding loop gains flexibility

2. Key residues are conserved in DHFR across 36 species

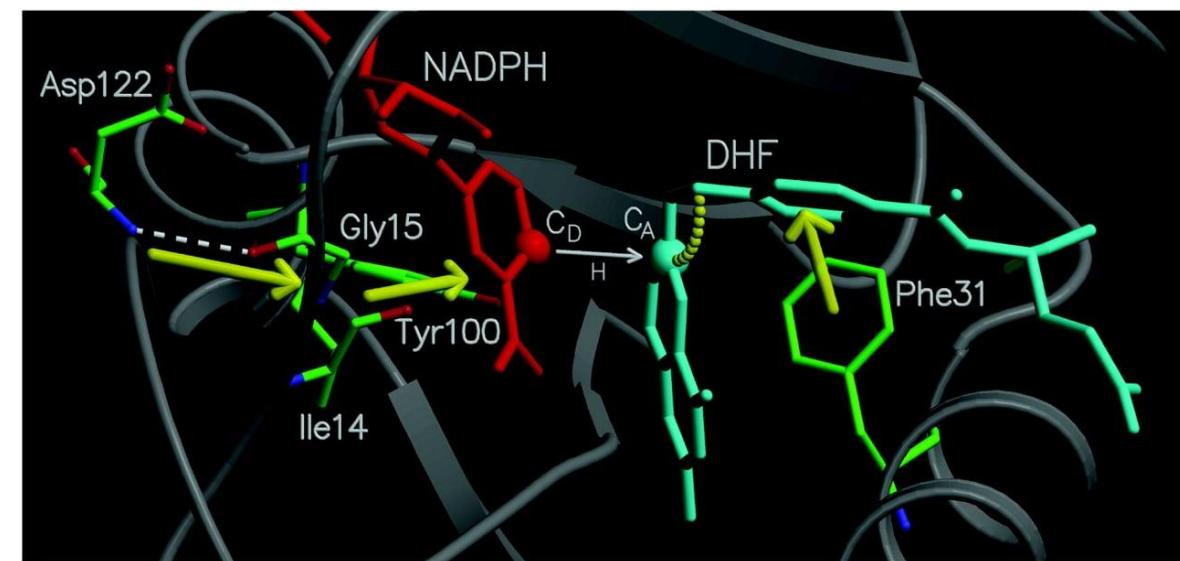
Wright Biochemistry, 2001, 9846



3. Mutagenesis of these residues decreases catalytic function in a nonadditive manner

e.g. M42F + G121A exhibits a 20-fold nonadditive effect on rate

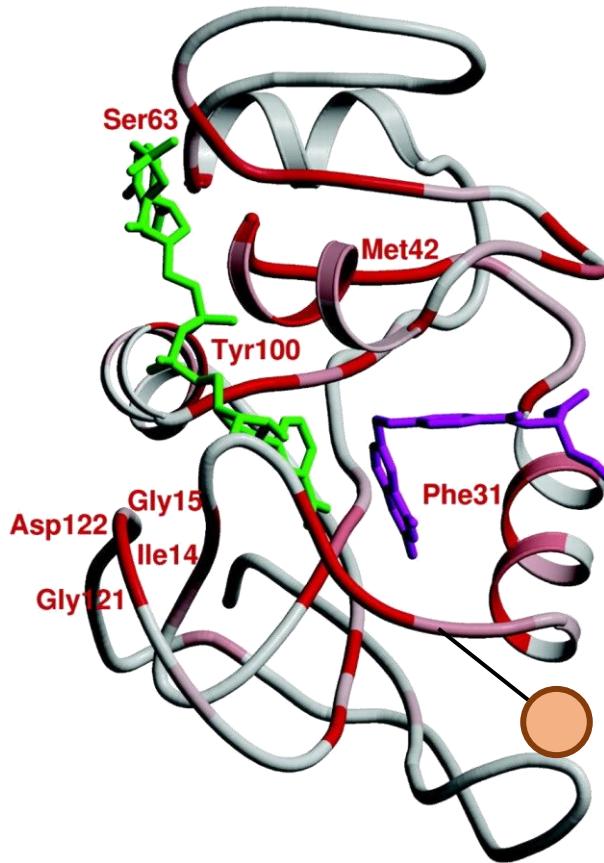
4. QM/MM MD identifies a network of coupled promoting motions



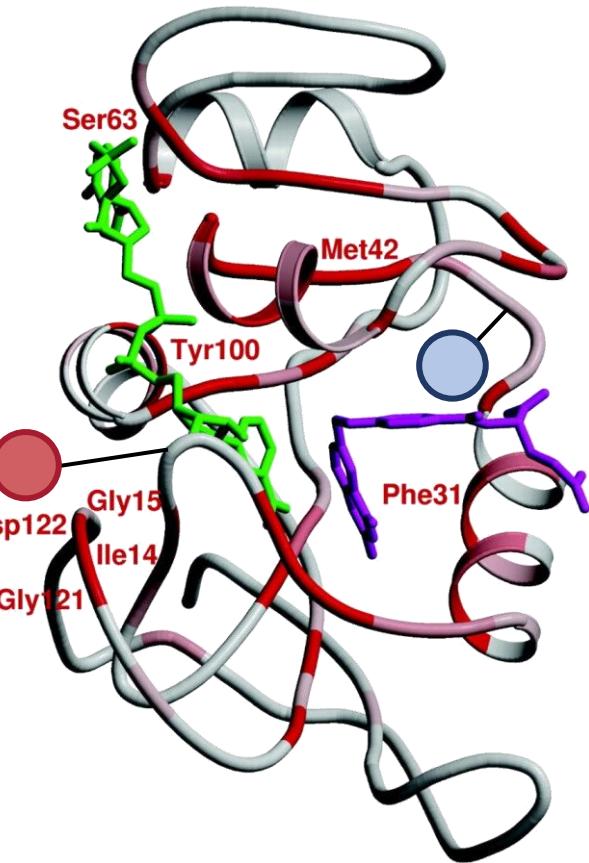
Equilibrium, thermally averaged motions are *not* coupled to bond formation, but instead set up an electrostatically "conducive" AS

Fluorescence labeling studies suggest conformations coupled to catalysis

Construct A (fluorescent probe)



Construct B (FRET pair)



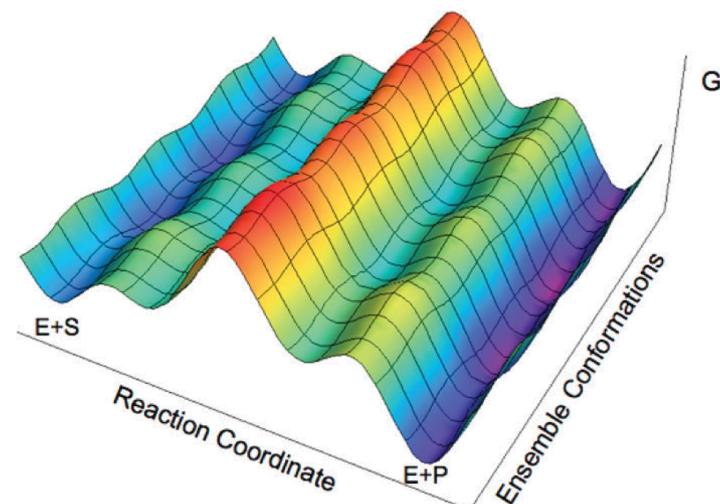
For each construct, further introduce G121V mutation that is known to affect protein motion and rate of reaction

hydride transfer fluorescence quenching

construct	k_{ht} (s^{-1})	$k_{ensemble}$ (s^{-1})	KIE	$k_{single molecule}$ (s^{-1})
A ^b	146	130	2.8	180
A, G121V ^c	1.3	33	no	ND ^e
B ^d	210	280	no	210
B, G121V ^d	3.5	154	ND ^e	145

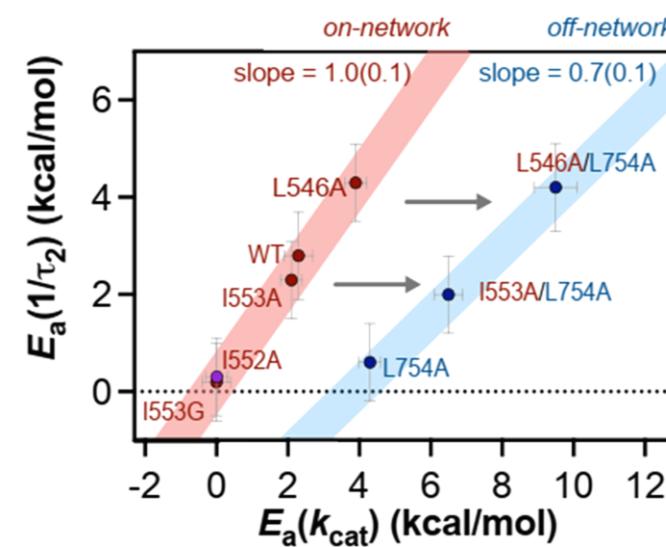
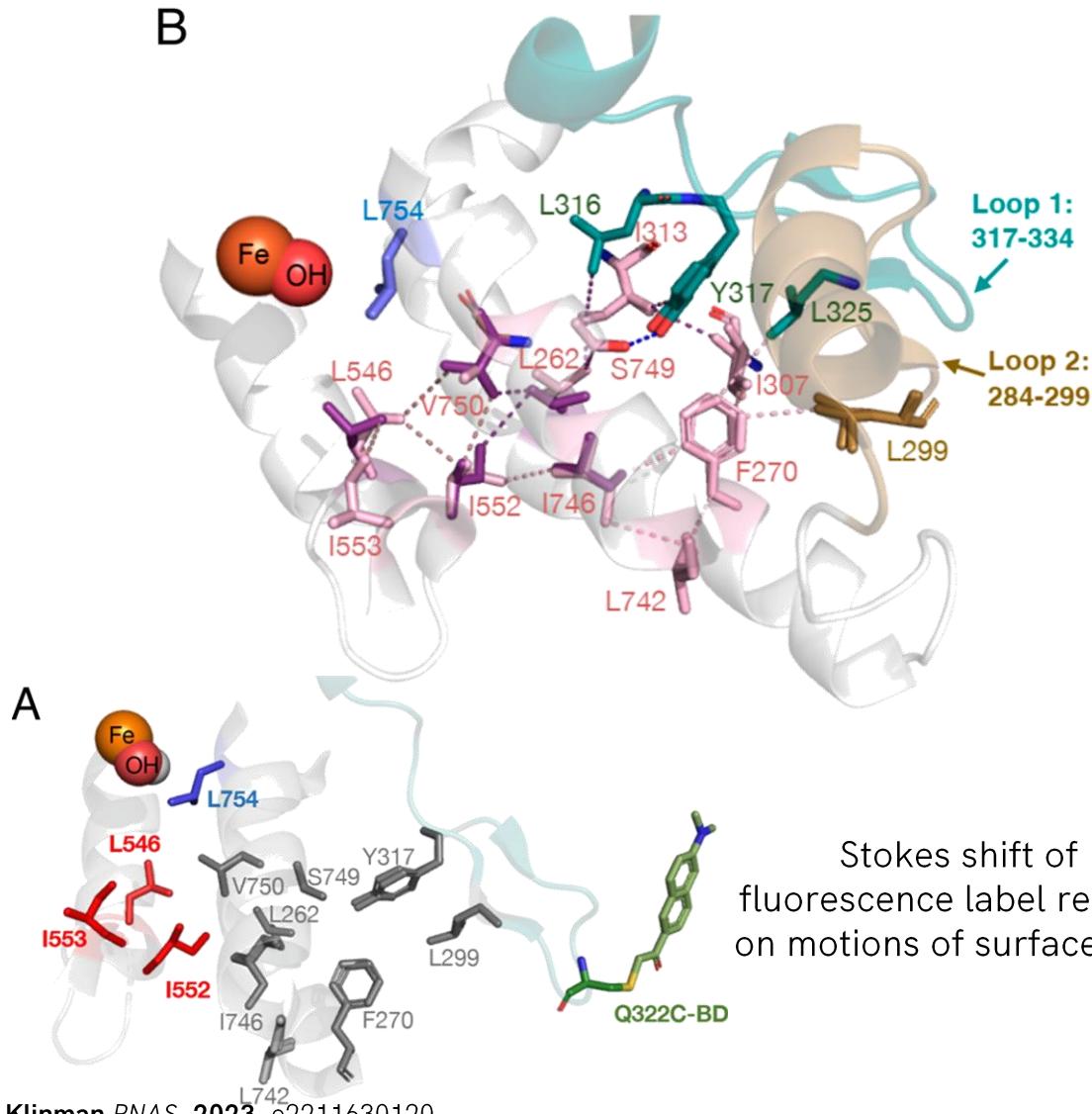
Interpretation

1. In native constructs, fluorescence (dynamics) agrees with, and is coupled to, hydride transfer
2. In mutants, fluorescence becomes decoupled from HT.
3. The three methods of detection are measuring different conformational changes, and mutation causes different conformational changes to be populated and coupled to HT.



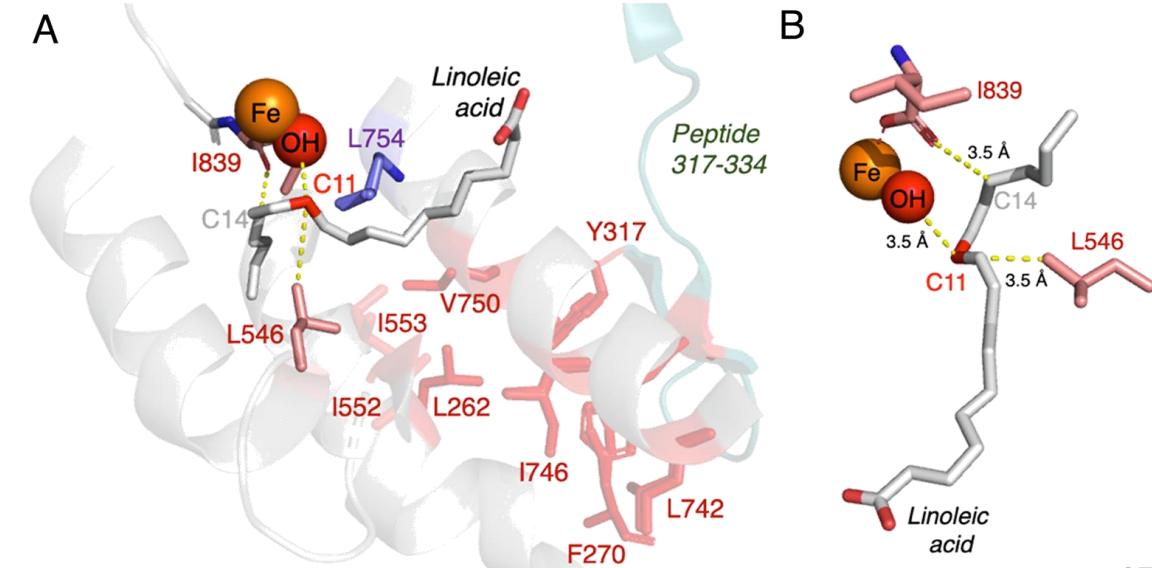
Soybean lipoxygenase exhibits a network of distal protein motions coupled to catalysis

Prior studies identified thermally activated **loop 1** and a network of **residues** leading into the active site

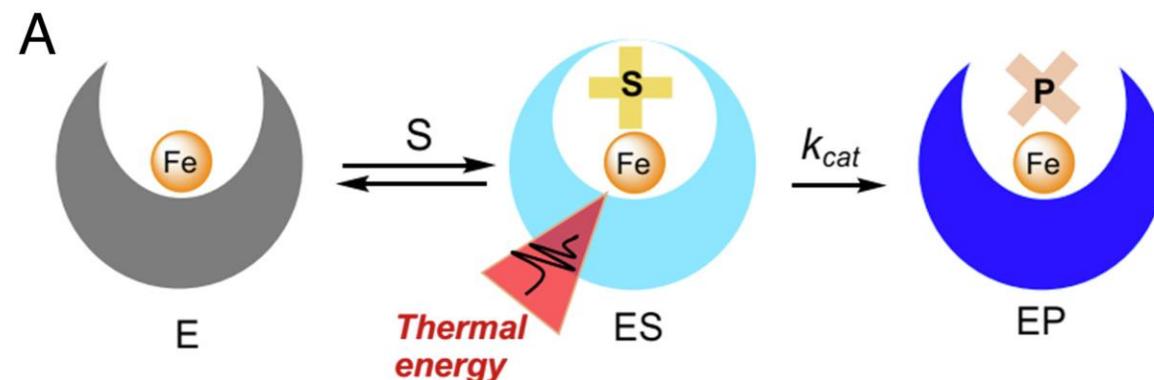


Activation barriers of Stokes shift dynamics (ns) and the chemical step (ms) agree across a series of mutations of the key residues

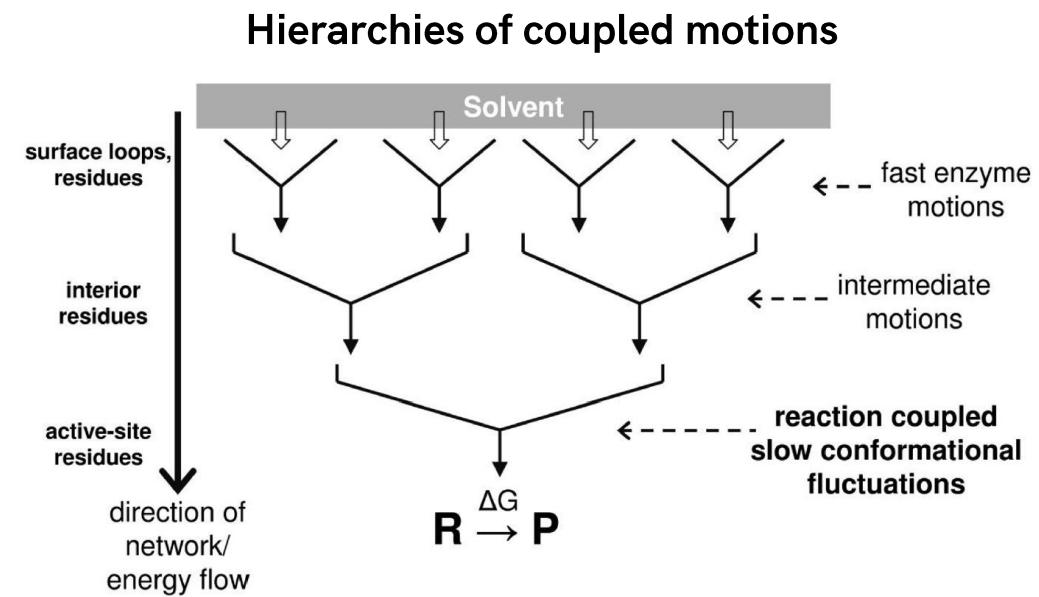
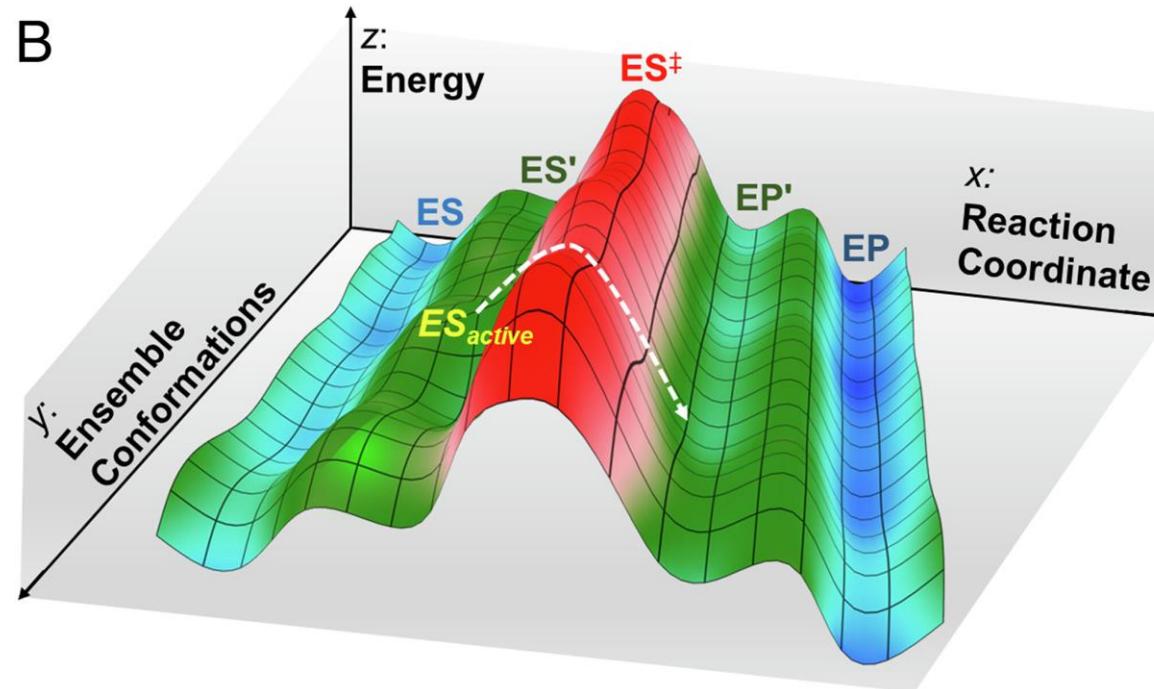
30-Å network culminates in L546 that “nudges” reactive C11 into OH



Networks of promoting motions transmit thermal energy from bulk solvent into the AS



Active sites protect substrate from thermal collisions with solvent, and instead use “privileged thermal conduits” in the protein scaffold to overcome the remaining 5-10 kcal mol⁻¹ barriers in a directed manner.



Overview

~~How do enzymes work?~~

~~What is the origin of large enzymatic catalytic proficiencies?~~

How do enzymes promote reactions with great rate acceleration *relative to the same mechanism in water?*
by GSD or TSS

How do enzymes achieve differential stabilization of the TS relative to the GS?
using electrostatic fields generated by a preorganized active site

What physical processes lead to the formation of electrostatically preorganized active site for barrier crossing?
coupled dynamic motions facilitate conformational sampling and conduct thermal energy from the surface

Enzymes use **electrostatics** and **dynamics** to promote difficult reactions with astronomical efficiency,
supported by their large protein scaffolds.

Overview

Open questions

1. Can we improve protein function by deliberately manipulating electric fields and dynamic conformational sampling?
2. Are these models for enzyme catalysis unifying beyond the few model systems studied?
3. (How) has electrostatics and dynamics biased the course of biochemical evolution?
4. Can we ever design minimal molecules that are competitive with enzymes? :’

What we have not covered

1. how enzymes evolved function (change in mechanism, tunneling, etc.)
2. how enzymes evolved specificity
3. how enzyme promiscuity/generality developed
4. how allostery or cooperativity works (clue: dynamics!)