ACS Catalysis

pubs.acs.org/acscatalysis Review

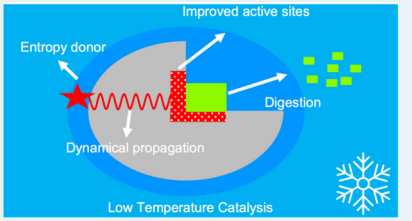
Rational Engineering of Enzymes for Enhanced Cold Activity

Weigao Wang and James Swartz*

Cite This: ACS Catal. 2024, 14, 12518−12528 Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Temperature-dependent evolution of enzymes allowed for biological adaptation to the wide range of temperatures found across Earth's ecological niches. Adaptation of enzyme activity to low temperatures presents the challenge of providing efficient catalysis with a relative deficiency of thermal energy, while adaptation to high temperatures requires increased thermostability. Evolved and engineered solutions to increasing both hot and cold activity have multiple advantages for industrial applications. Here, we review studies toward engineering important activities for lower temperature catalysis while categorizing three different mechanisms reported to underlie enzymatic cold adaptation: structural flexibility, surface softness, and allosteric dynamics. Further, we analyze the relative efficacy of these mechanisms and conclude that modifying allosteric structural dynamics most frequently leads to the cold temperature adaptation of enzymes. Finally, we offer guidance for rational improvement of low temperature enzymatic activities using allostery pathway mapping tools.



KEYWORDS: *low temperature catalysis, temperature adaptation, structural flexibility, surface softness, allosteric dynamics, entropy engineering, rational design*

INTRODUCTION

The concept of green chemistry was coined in the 1990s and was embodied in 12 principles which collectively propose a mild, safe, and green approach toward conserving energy and resources.[1] Biocatalysis in the context of green chemistry has the potential to be both environmentally and economically superior to conventional technologies. In this pursuit the catalytic performance of individual enzymes play an important role in determining the energy conservation and economic effectiveness of such biological processes. Since enzymes used in synthetic biology pathways have temperature dependent activities, the mild conditions principle sought for green chemistry are often challenging to meet. Therefore, engineering robust enzymes that can be functional through a wide range of temperatures could provide more productive, less expensive, and more robust industrial processes.

Extremophiles are microorganisms that live in extreme conditions of temperature, pressure, pH, salinity, radiation and water activity. Thriving in these environments is often aided by enzymes that have evolved to function effectively in such environments. Their unique properties have been exploited across the food, chemical and pharmaceutical industries.[2] While much of the focus is on thermophiles due to favorable operating conditions (e.g., elevated reaction rates at higher temperatures, prevention of contamination, long lifetime due to thermostability), psychrophiles have evolved unique enzymes with catalytic properties that enables fast reaction rates in the relative absence of thermal activation energy (Figure 1).[3] Effective catalysis at these lower temperatures can offer lower energy consumption and improved process performance.

Understanding the evolutionary adaptations that enable effective enzyme function in extreme conditions is critical to the development of rational design principles for improving biocatalyst usefulness (Table 1). In this review, we focus on theories that aim to explain the structural characteristics influencing enzymatic activation energy reduction to improve low temperature kinetics. Through a comprehensive examination of each theory's rationale, we have determined that the dynamic allostery theory provides a better understanding of the relationship between structural features and activation energies, specifically in relation to low temperature adaptation. Moreover, we also discuss the significance of retaining enzyme dynamics for low temperature enzyme catalysis. Based on our analysis, we propose that dynamic allostery engineering could serve as a potential strategy for lowering the activation barrier of catalysis and improving enzyme performance at lower temperatures. Additionally, we have reviewed various tools for allosteric analysis that can be utilized to identify dynamic allostery pathways. Understanding these pathways can be instrumental in guiding the rational engineering of low temperature enzymatic adaptations. We suggest that such

Received: May 8, 2024
Revised: July 19, 2024
Accepted: July 22, 2024
Published: August 6, 2024

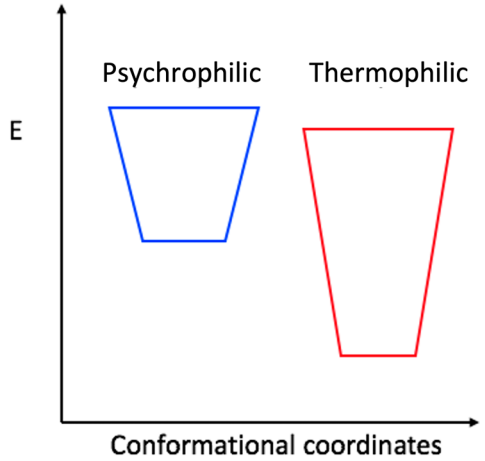


Figure 1. Proposed model of folding funnels for psychrophilic and thermophilic enzymes. In these schematic energy landscapes, the free energy of folding or unfolding (E) is represented as a function of the conformational diversity. Reproduced with permission from ref 20. Copyright 2003 ASBMB.[20]

approaches can enhance catalytic performance and lower production cost within the biomanufacturing industry.

THERMOPHILIC ENZYME STRUCTURAL FEATURES

Thermophiles optimally grow at high temperatures (between 45 and 121 °C) and have been the major focus of extremophile enzyme research, as indicated by several excellent reviews.[3−5] The most common reason invoked for using thermophilic enzymes is reduced enzyme costs due to higher activities and improved stability. Catalysis at elevated temperatures requires thermophilic enzymes to retain functional activity and structural integrity in spite of potentially elevated molecular dynamics. The stability of thermophilic enzymes can be attributed to increased Coulombic interactions of charged surface residues, larger hydrophobic cores, shortened surface loops, disulfide bonds, protein−protein stability, cooperativity and more stable oligomerization.[6,7] Increased surface charge can also reduce the interactions between water molecules and the protein surface to possibly slow the penetration of water molecules into the hydrophobic cores.[8] This later concept taken more broadly has been applied to improve the thermal stability of enzymes by replacing native surface residues with arginine and proline.[9,10] Increased size of hydrophobic cores is also believed to exclude water and decrease the flexibility of the internal structures.[6] Based on these structural features, thermophilic enzymes are generally considered to be rigidified thereby increasing resistance to structural plasticity as a mechanism for enhancing thermal stability. However, thermal stability is not always imparted by increased rigidity. Neutron scattering and NMR spectroscopy measurements on a thermophilic cytochrome P450 (CYP119) and its mesophilic counterpart (CYP101A1) showed that thermophilic CYP119 is actually more flexible. The increased thermal stability was instead due to reduced entropic gain and increased energy requirements for unfolding toward denaturation.[11] Therefore, the terms "rigidity" and "flexibility" overlook the relative motion of stable regions in the thermostable proteins as well as entropic contributions to stability enhancement.[12]

PSYCHROPHILIC ENZYME STRUCTURAL FEATURES

Psychrophilic enzymes are found in cold-adapted microorganisms. Their applications in food processing, stain removal, the pulp and paper industry provide advantages by decreasing energy consumption due to lower operating temperatures.[13] In many cases, the specific activities of psychrophilic enzymes at low temperatures are comparable to those of mesophilic and thermophilic enzymes at their respective optimal temperatures.[14,15] As mentioned, evolutionary adaptations of psychrophilic enzymes enable intrinsically better catalytic rates in spite of lower available thermal energy. It is therefore generally believed that psychrophilic enzymes have evolved with increased flexibility and decreased stability to provide increased intramolecular dynamics at lower temperatures.[16] This proposed inverse relationship between flexibility and stability in psychrophilic enzymes has long been attributed to structural features such as the increased frequency of alanine, aspartic acid, serine and threonine; the generally increased proportion of small and neutral residues; and the increased percentage of α-helical structure.[13,17,18] However, such structural differences sometimes are not present between orthologous psychrophilic-mesophilic pairs of enzymes.[19] Additionally, the flexibility-stability exclusivity theory is challenged by the failure to find a strict inverse relation between stability and low temperature activity. It has been proposed that the trade-off between increasing low temperature activity while decreasing structural stability could be attributed to a smaller selective pressure toward stability allowing this attribute to weaken in the cold environment.[15] Despite the fallacy of flexibility-stability exclusivity, it is true that most naturally occurring psychrophilic enzymes are more heat labile due to a higher entropy gain ($\Delta S^{\ddagger}$) than their mesophilic homologues during unfolding. This is consistent with the folding funnel theory.[20] According to this model, the height of the funnel is deduced from the determination of the conformational stabilities. A thermophilic enzyme would have more stable intermediates, so its funnel depth is higher than for psychrophilic enzymes which have fewer intermediates in the unfolding process.[18,21,22]

To perform actively at the low temperatures, psychrophilic enzymes appear to have larger active sites to counteract the slower substrate diffusion rates that facilitate the binding of ligand as well as product release. This also explains why psychrophilic enzymes often have more related specification

Table 1. Summary of Cold Adaptation Theories

theories	key ideas
active site flexibility	this theory proposes that the active site flexibility explains the cold adaptation of psychrophilic enzymes because the flexibility of active sites narrow the free energy gap between the ground state and transition state
surface softness	this theory proposes that as the constraints on the psychrophilic enzyme was moving deeper from the surface, its thermodynamic parameters gradually become mesophilic enzyme type parameters
dynamic allostery	this theory proposes that enzyme catalytic core residues are allosterically connected with the surface residues or domains, and the dynamics can be propagated into the catalytic core to tune the activation entropy change and thus modifying the catalytic efficiency

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

Table 2. Activation Parameters for Psychrophilic and Mesophilic Enzymes[5,47,23−25]

enzyme	source	T (°C)	k_{cat} (s^{-1})	$\Delta G^{‡}$ (kJ/mol)	$\Delta H^{‡}$ (kJ/mol)	$T\Delta S^{‡}$ (kJ/mol)
trypsin	psychrophilic	5	82.0	57.5	33.0	−24.5
	mesophilic		27.77	60.2	53.9	−6.2
trypsin	psychrophilic	15	0.97	70.5	40.6	−29.9
	mesophilic		0.68	71.4	82.6	11.3
amylase	psychrophilic	15	495.2	55.6	73.6	18.0
	mesophilic		90.5	59.7	99.6	39.9
subtilisin	psychrophilic	15	25.4	62.7	36	−26.7
	mesophilic		5.4	66.4	46	−20.4
glucose-6-phosphatase	psychrophilic	0	106.5	56.1	36.8	−19.3
	mesophilic		42.4	58.2	51.9	−6.3
xylanase	psychrophilic	5	14.8	61.7	45.4	−16.3
	mesophilic		4.9	64.3	49.9	−14.4
chitobiase	psychrophilic	15	98.0	59.5	44.7	−14.8
	mesophilic		18.0	63.5	71.5	8.0
chitinase a	psychrophilic	15	1.7	69.2	60.2	−9.0
	mesophilic		3.9	67.2	74.3	7.1
lactate dehydrogenase	psychrophilic	20	53.1	5.8	−10.1	−15.0
	mesophilic		6.0	7.0	−10.2	−10.0
elastase	psychrophilic	22	NA[a]	18.0	4.6	−13.4
	mesophilic		NA	19.2	17.2	−2.0
endonuclease I	psychrophilic	5	NA	62.8	33.4	−29.4
	mesophilic		NA	67.9	74.0	6.1
lysozyme	psychrophilic	25	NA	45.1	31.9	−13.2
	mesophilic		NA	46.2	49.4	3.2

[a]Not available.

and larger K_m values at low temperatures when compared with their mesophilic/thermophilic homologues.[3,21]

Even though there is comprehensive research concerning the structural features of extremophile enzymes, a clear and consistent path remains to be revealed for engineering mesophilic or thermophilic enzymes toward increased cold activity, thereby creating more robust and energy efficient enzymes. The complicated structure and multiplicity of internal interactions between residues make it challenging to sufficiently characterize enzyme flexibility for improved psychrophilic enzyme engineering. In this review, we evaluate proposed design approaches and suggest future directions for rational design toward increased cold activity.

ENGINEERING OF ENZYME ACTIVE SITE FLEXIBILITY

As mentioned above, psychrophilic enzymes need to provide the structural kinetics necessary for the enzymatic reaction to proceed at low temperatures. Comparing the thermodynamic parameters of psychrophilic and mesophilic analogs indicates that the activation energy(ΔG) for the psychrophilic enzymes has generally decreased (Table 2).[3] According to the Arrhenius equation

$$k_{cat} = Ae^{-(\Delta H - T\Delta S)/RT}$$

a decreased ΔH contributes to an increase in k_{cat}, while a smaller ΔS exerts a negative effect. The reason why k_{cat} for the psychrophilic enzymes is higher than the mesophilic/thermophilic homologues is that the ΔH decrease is larger than the impact of the entropic change. The net effect decreases activation energy $\Delta G(\Delta G = \Delta H - T\Delta S)$. With regard to structure, psychrophilic enzymes need to maintain locally stable domains while increasing the flexibility of the active sites to reduce the counter effect of ΔS.[3,18] The increased flexibility in the reactant state would imply a larger configurational space and result in a more negative activation entropy if the configuration of the active site in the transition state still remains approximately the same as in the mesophilic enzyme.

This hypothesis is corroborated by the characterization of lactate dehydrogenase A4 from Antarctic *notothenioid teleosts* and citrate synthase from *Antarctic bacterium* where both of them showed local flexibility only in a single domain of the enzyme, as opposed to globally distributed flexibility.[26,27] Similarly, the fact that psychrophilic enzymes lose activity before their structure is unfolded also indicates the uneven

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

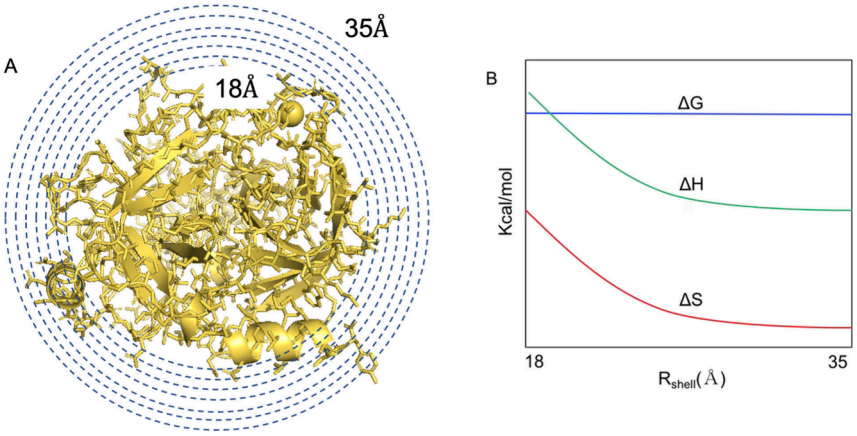


Figure 2. Effects on thermodynamic activation parameters of systematic variation of AST protein surface flexibility. (A) Simulation sphere where solute atoms between R_{shell} 18 and 35 Å are restrained with a force constant of 100 kcal/(mol Å^2). (B) Thermodynamic activation parameters (300 K) for the systems restrained in A, showing that the parameters for cold-active trypsin are gradually converted to those of the warm-adapted trypsin. Reproduced with permission from ref 46. Copyright 2016 National Academy of Sciences.[46]

distribution of the forces stabilizing the active sites being weaker than those of the other domains.[21] Furthermore, differential scanning calorimetry (DSC) studies of the multidomain protein phosphoglycerate kinases (PGK) showed that the stable domains of the cold-adapted PGK exhibited higher stability compared with its mesophilic counterparts, which suggests that the thermotolerant protein does not necessarily have higher stability. Accordingly, it was proposed that localized stability and flexibility can be evolved separately, and engineering the active sites for improved flexibility could be a promising strategy for increasing activity at lower temperatures or even for engineering robust enzymes with high activity across a wide-range of temperatures.[21,28,29]

Success with engineering local active site flexibility has been reported in several studies so far. An earlier example of cold adaptation focused on a mesophilic subtilisin-like protease. After several rounds of random mutagenesis and recombination, a mutant was generated with higher k_{cat}/K_M at lower temperatures. Mutations were located both close to and far from the active site, and it was suggested that weakening the hydrogen bonds around the active site increased local mobility to confer cold-adaptation functionality.[15] A recent study on *Bacillus agaradherans* cellulase Cel5A found that the decreasing of hydrogen bond interactions between loops in which catalytic residues are located reduced the activation enthalpy and activation energy. The mutants provide higher activity at both cold and warm temperatures. The cold enzyme-like features were concluded to be caused by increased fluctuation near the active sites.[30] In this research, multiple sequence alignment (MSA) between Cel5A and its mesophilic and psychrophilic orthologues showed high overall identity with the exception that residues near the active sites are modified. In the study of a thermophilic lipase, Wang et al. tuned the activation enthalpy and entropy values of this enzyme to decrease the activation energy barrier by rationally weakening the hydrogen bonds around the active site. The thermophilic mutants exhibited increased specific activities at lower temperatures, and surprisingly, improved thermotolerance compared to the wild type enzyme.[31] Similarly, Gault et al. were able to lower the optimal temperature of a thermophilic α-chymotrypsin by adding perchlorate salts found on Mars. It was concluded that the perchlorate salts increased the conformational flexibility of the enzyme and made the formation of transition state complexes much easier.[32]

However, it may not be accurate to directly relate the increased flexibility or ground state destabilization to the increase in catalytic rates. Warshel suggests that increasing the ground state energy without changing the transition state energy will alter k_{cat} and K_m but still leave k_{cat}/K_m constant. In this case, the actual effect of increasing ground state motions is anticatalytic.[33,34] The smaller T$\triangle$S in psychrophilic enzymes only indicates the difference between the space available for thermal motions in the reactant state and transition states, not the difference between the active site motions in psychrophilic enzymes and mesophilic/thermophilic enzymes.[33] Furthermore, it was also concluded that the reduction of the reorganization energy actually plays a role in enzyme catalysis. Therefore, understanding what influences the reorganization energy is meaningful in understanding the temperature adaptation.[33]

It should be noted that not all the residues around the active sites have been systematically varied in many projects. Enzymes are grouped into the same superfamily when they share similar catalytic activities, sequence motifs and other conserved features like the overall organization of the active sites, conservation of certain active site residues, and common reaction intermediates.[35] Most extremophile enzymes share active sites with similar residues even when their temperature optima are different. Additionally, linear sequences beginning with amino acids like alanine and glycine are generally believed to be more flexible compared with those with other amino acids, but the indiscriminate introduction of flexibility around the active sites may impair the enzymatic activity significantly. This is because the positions of many of the residues close to the active sites may play important roles in maintaining the structural integrity of the catalytic core providing the channel for the substrate to approach in the catalytic cavity.[36−40] This was also observed in our study of a thermophilic lipase in which amino acid changes near the active sites often impaired the activity when they were mutated to alanine or glycine.[41] Furthermore, mutations near the active sites may lower the energetic stability provided by hydrophobic residues in the internal core of the enzyme, thus destabilizing the structure and causing the protein to aggregate as it explores potentially unfolded forms.[39] Lastly, allosteric changes across the enzyme

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

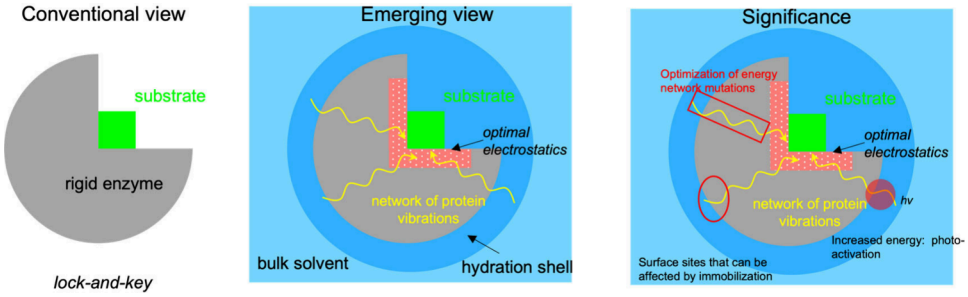


Figure 3. Interconnection among protein structure, dynamics, and function has significance for improving the catalytic efficiency of enzymes. The active site region provides an environment with optimal electrostatics to promote the catalyzed reaction. Several enzymes contain conserved networks of residues that connect surface regions to the active site. Reproduced with permission from ref 67. Copyright 2018 American Chemical Society.[67]

structures can transmit the dynamics from one position to another,[39,42,43] and introduced flexibility around the active sites may be hard to localize and may also be propagated causing an overall structural change leading to instability.

PROTEIN SURFACE SOFTNESS

Even though the flexibility around enzyme active sites has been successfully engineered for temperature adaptation, directly relating temperature adaptation to enzyme active site flexibility is not always supported by experimental observations. Some enzymes having different optimal temperatures showed identical flexibility across the whole structure.[44,45] Computational simulations of bovine trypsin (BT) and Antarctic salmon trypsin (AST) structural feature revealed that significant flexibility is only conferred in two cold-adapted conserved residues on the protein surface removed from the active site in AST (Figure 2). This is the only structural difference to explain its cold adaptation.[45] Such conserved surface residues can be found in all psychrophilic trypsins. Additionally, mutations at these two conserved residues (N/Y97 and S/D150) individually changed the temperature adaptation of BT and AST in the opposite direction by tuning the activation energy, enthalpy and entropy values. The cold adaptation of AST was attributed to the less polar residues and the disruption of hydrogen bonding with water.[45]

The protein surface flexibility on AST was further studied using the simulation to establish a restraint in the enzyme at various depths from the protein surface.[19,46,47] The activation enthalpy ΔH and activation entropy ΔS gradually increased when the restraint was installed much deeper from the protein surface toward the internal core, despite the activation energy $\triangle G$ remaining relatively stable.[46,48] The restraint on the key residues in AST produced similar thermodynamic properties as for the residue 97 mutant. Similarly, the increased mobility in the surface loops of a cold-adapted elastase also suggest its importance in tuning temperature adaptation when compared with the mesophilic analog.[49] These two studies support the hypothesis that structural restraints that rigidify enzyme surface softness lead to warm adaptation, and that surface softness contributes to cold adaptation. This was further supported when the rational engineering of porcine pancreatic elastase increased the k_{cat} values at low temperatures after replacing its surface residues with the conserved residues in salmon pancreatic elastase (SPE).[50]

The surface softness theory is thus able to explain the temperature adaptation of some enzymes and also provides a new direction for cold activity engineering. Protein surface loops are, therefore, regarded as new hotspots for altering the temperature dependence of enzymes. However, this theory is still disputable for explaining enzyme cold temperature adaptation. First, not all the residues on the protein surface are equally important in temperature adaptation. Only about half of the residue changes in PPE(porcine pancreatic elastase) increased low temperature activities when mutated into conserved residues from SPE. Moreover, the oligomerization of the short chain dehydrogenase from *Psychrobacter arcticus* reduced the mobility of several residues in each monomer but made the tetramer more cold-adapted, which seems to contradict the surface softness theory.[51] Further, a cold adapted esterase, EstN7, has more rigidity features (proline residues, salt bridge, well packed core and shorter dynamic caps) compared with the mesophilic and thermophilic relatives.[52] Therefore, another theory is needed to better explain how structure features influnces temperature adaptation.

When simulations of AST and BT structural rigidity were revisited, the high flexibility was mainly contributed by two specific residues on surface loops, suggesting that these evolutionarily conserved hotspots may play an important role in tuning temperature adaptations.[45] This makes sense because the evolutionarily conserved residues tend to stimulate the formation networks called "sectors" that are important in modulating the enzyme function.[53,54] Considering that the internal motions of the catalytic residues were linked with the motions of protein surface loop regions, the altered thermodynamic properties of AST caused by the restraints on the surface softness might actually be attributed to the disturbed motion propagations from the surface loops to the residues around the active sites.[55]

DYNAMIC ALLOSTERY

Allostery is generally described as an information coupling phenomenon between sites in a protein. It is induced by a local perturbation and regulates the protein's structure, dynamics and functions via a large-scale transmission (Figure 3).[42,56] Allostery was recognized as being encoded in protein sequences by evolution, and the allosteric coupling residues have been identified as covaried in an evolutionary approach.[57,58] Some of the networks formed by covaried residues in the evolution process were also associated with propagating the flexibility and promoting the catalysis step by connecting the motions on the surface loops to the active sites.[55,59,60] These allosteric networks for motion propagation share common features. First, networks connect surface loop

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

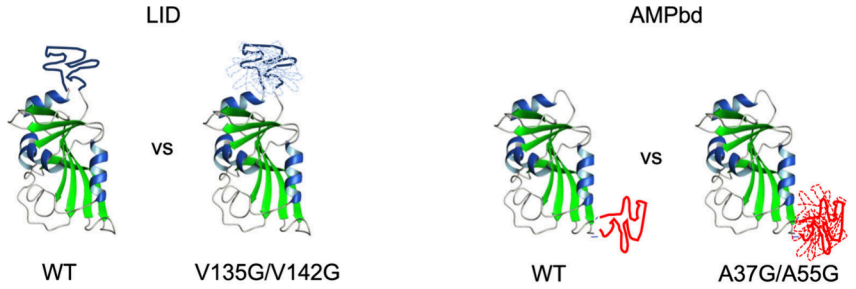


Figure 4. Domain structure of *E. coli* adenylate kinase and sites of allosteric entropy-tuning mutations. Entropy-enhancing Gly mutations. Changes from Val to Gly in the LID domain (left) and Ala to Gly in the AMPbd domain (right) are visually quantified by amounts of locally unfolded conformations. WT, wild type. Reproduced with permission from ref 69. Copyright 2018 Springer Nature.[69]

regions to conserved active-site residues that make direct contacts with the substrates over a long distance through hydrogen bonds or hydrophobic interactions.[55,59,61−64] Second, bulk solvent fluctuations, long side chain residues in the solvation shell, or nearby loop motions are able to drive internal protein dynamics through those networks.[52,55,60,65,66] Third, the time scale of these network motions range from picoseconds to milliseconds and is coupled to the reaction steps.[67] Experimental techniques have enabled the discovery of a series of these networks in a series of enzymes whose surface residue motions are functionally relevant to active site catalysis.[55,59,60,62,66,67] In the study of dihydrofolate reductase-(DHFR), Agarwal et al. has characterized a portion of the dynamic network connecting the conserved residues on the surface of the protein to the active site, having motion durations ranging from femtosecond to millisecond.[62] These networks thermodynamically couple the hydration shell and bulk solvent with the catalyzed reaction. The discovery of these enzyme energy networks allows the development of new strategies for increasing the catalytic efficiency by enhancing the energy flow through these networks using mutational optimization.

Impairment of the conserved surface residues motion decreases the DHFR hydrate transfer rate significantly.[62] Another study by Wang et al. found that the decrease of the hydrate transfer rate is caused by a decrease of the network motion and an increase in preorganization(smaller $T\triangle S$ value).[31] Agarwal proposed a hierarchy model to explain how the long-distance network connects the surface residue motions to the active site.[67] In this model, fast motions located in the surface and loop residues are driven by the interactions with the solvent and by thermodynamic fluctuations. These motions are then propagated into the interior residues to induce intermediate motions and stimulate additional enzyme conformational fluctuations. Eventually, propagated conformational fluctuations change the electrostatic environment of the active site residues, stabilize the interactions with the substrate, overcome the activation energy barrier, and stimulate catalysis.[67] This motion propagation process includes energy collection(fast motions), energy transfer via motion networks, and stimulation of the active site.[67]

Several studies have described the importance of such internal transmissions in adjusting the enzyme catalysis under various temperatures. Dong et al. compared the structural differences of malate dehydrogenases between five thermally adapted congeners and noticed two regions, MR1 and MR2, in cold adapted dehydrogenases that exhibit higher flexibilities and are responsible for improved protein catalysis and substrate binding. Interestingly, mutations outside these two regions could still make the enzyme more cold-adapted.[68] The conformational transmissions from the relatively flexible surface residues to the catalytic residues and substrate binding sites regulated the temperature adaptation of enzymes.[65] In an adenylate kinase study, mutation at the AMP-binding domain increased the k_{cat} at every temperature by decreasing the activation entropy without interfering with the activation enthalpy. This was achieved by the dynamic allostery originating from conformational entropy-enhancing mutations on the enzyme surface (Figure 4).[69] These mutations modified the distribution of folded versus unfolded conformations, enhancing the stability of the locally unfolded state in the mutant AMPbd. This alteration effectively reduced the activation barrier for product release, consequently leading to an acceleration in the reaction rate.[69] Furthermore, the distinct impacts of mutations on substrate binding and product release serve as evidence for segregated roles of allostery within the enzyme's functionality. The introduction of the less voluminous glycine at the mutation sites was observed to elevate the proportion of the unfolded state within the AMP-binding domain by disrupting an α-helix. This domain was spatially separated from the rigid core domain with only two loops connecting them, thus producing entropy and propagating the dynamic motion through the hinging loops to the core domain. Analogous findings were also observed in the investigation of α-amylase orthologues, wherein the influence of flexible loop L7 was identified as a contributing factor to the psychrophilic traits.[70]

The finding that the allosteric dynamics generated by the AMP-binding domain of adenylate kinase can affect its temperature adaptation provides a new direction for the rational design of cold activity in enzymes. However, several questions need to be addressed to rationally make enzymes more cold-adapted via allostery. First of all, not all protein surface residues can serve as hotspots for regulating the temperature adaptation because they are not equivalent entropy donors. Hacisuleyman et al. investigated ubiquitin and classified residues into entropy donors and acceptors in allosteric entropy transfer processes (Figure 5).[71] Entropy donors are generally located in the nonsecondary structures on the protein surface while entropy acceptors are in the secondary structure inside the protein core. The entropy transfer between donors and acceptors is unidirectional.[71]

Therefore, the surface softness theory explaining enzyme cold temperature adaptation could also be a reflection of the entropy increase when certain mutations on the protein surface made BT more cold-adapted. The observation that applying different depths of constraint to the enzyme in simulations

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

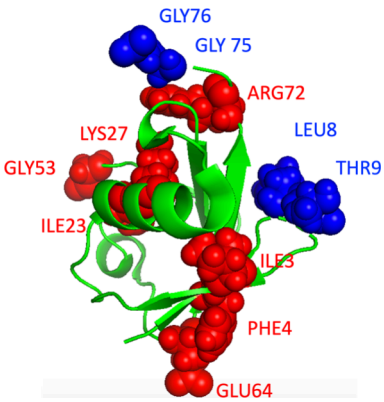


Figure 5. Structure of ubiquitin, residues that are colored in red are entropy acceptors and residues that are colored in blue are the entropy donors. Reproduced with permission from ref 71. Copyright 2017 Hacisuleyman, Erman.[71]

altered the balance between entropy and enthalpy as influencing cold adaptation might be attributed to the impacts of constraints on the entropy transfer from the surface entropy donors. Second, even though residues can act as entropy donors on the protein surface, not all of the dynamics can be propagated to the catalytic region to alter the energy barrier of enzyme catalysis. The disruption of the secondary structure in the LID domain of adenylate kinase only changed the binding affinity of the substrate without affecting the k_{cat} value,[69] while the effect of entropic allostery has been found to affect the binding affinity, stability, and catalytic rate in other proteins.[11,72−76]

A study of the Kemp eliminase variants might give us some hints as to why entropic dynamics can improve catalytic efficiency. The structural comparison between 1A53−2 and 1A53−2.5 variants of Kemp eliminase showed that the latter variant has larger and bulkier residues.[77] The absence of these larger residues in 1A53−2 creates narrow solvent channels into the active site and increase the enthalpic cost in the transition from ES'(relaxed reactant state) to ES(contact reactant state). The water cavities also render the ES' state more entropically strained.[70] In other words, the replacement of smaller residues by bulkier residues excludes solvent from the active site and maintains the ES' state at a higher entropy level. It is worth noting that the increase in low temperature activity of EstN7 was also attributed to the negatively charged residues on the protein surface which form interactions with water and offset the cold-denaturation caused by decreased dynamics.[52]

■ DYNAMICS AND CATALYSIS DEBATE

As previously discussed, the concept of allosteric dynamics evolution presents a more plausible explanation for cold adaptation, and it offers a viable avenue for systematically enhancing the cold activity of enzymes through deliberate manipulation. Nevertheless, further investigation is required to elucidate the mechanisms by which the propagated dynamics reach the active site to enhance catalytic rates. Establishing a direct correlation between dynamic processes and enzyme catalysis remains controversial. Watz et al. drew a noteworthy connection between protein dynamics and catalytic turnover rates when they recognized that the reduced activity of a hyperthermophilic adenylate kinase at ambient temperature resulted from a slower lid-opening rate for a large conformational change required for catalysis.[78−80] In a similar vein, Bhabha et al. generated a mutant in which enzyme active site fluctuations on the millisecond time scale were eliminated without perturbing the structural and electrostatic preorganization, but the loss of the Met20 loop dynamics significantly impaired the hydride transfer rate.[81] This study of DHFR hydride transfer revealed the important role of short length scale nonequilibrium dynamics in the intrinsic reaction.[82] According to Kohen, enzymatic systems seem to stiffen when close to transition-state and only enable dynamics along the reaction coordinate to occur selectively by restricting motions orthogonal to the chemical coordinate.[83] Enzymes could also evolve to use nonstatistical protein promoting vibrations to catalyze the bond activation event when all environmental motions are much faster than the barrier-crossing event.[60,83,84]

Conversely, some enzymatic studies on enzymes have presented evidence that challenges the theory linking dynamics to catalysis. A chimeric α-lactamase cTEM-17m mutant generated by Gobeil et al. exhibited comparable catalytic efficiency but significantly improved slow motions at the active site compared to wild type enzymes.[85] Tokuriki et al. proposed that the chemical modification step in the catalytic cycle is rate limiting because the active site is unlikely to be optimal for catalyzing the new activity, therefore enabling the tolerance of the millisecond dynamics change.[85,86] Warshel et al. scrutinized the dynamical proposal and concluded that there are no consistent studies that found significant dynamical contribution to enzyme catalysis.[87] The terminology "dynamics" used by experimentalists actually means thermally equilibrated dynamics rather than nonstatistical motions assumed by theoreticians.[83,87] No matter what the alternative definition of "dynamic" is (landscape search, entropy funnel or coupling between the conformational and chemical coordinates), the conformational motion does not bring "memory" to the chemical step, thus negating the important role of dynamics or entropy in catalysis.[87−89] Roca et al. calculated and compared the transmission coefficients of both enzymatic and reference reactions in solution and concluded that the increase of the rate constant caused by dynamical effects is much too small to explain the 10^7 increase in the overall rate.[90,91]

Allosteric dynamics evolution offers a promising approach for enhancing enzyme cold activity, but understanding how dynamic processes affect catalytic rates requires further investigation. Studies demonstrate correlations between protein dynamics and catalytic turnover rates, yet conflicting evidence challenges the significance of dynamics in enzyme catalysis. The debate over dynamics' role in enzyme catalysis persists, with evidence both supporting and refuting its significance. Clarifying the relationship between dynamics and enzyme function is crucial for biocatalysis and enzyme engineering. Continued research efforts are needed to resolve discrepancies and advance our understanding of dynamics' impact on enzyme activity.

■ ALLOSTERIC PATHWAYS

While the contribution of dynamics and entropy gain to catalysis remains a subject of ongoing debate, previous experiments and theoretical frameworks such as local flexibility, surface softness and allosteric dynamics indicate that dynamic components like loops and channels, as well as residues in their vicinity, might emerge as new focal points for enhancing enzyme performance at lower temperatures.[92] This concept aligns well with the practice of targeting these elements to fine-tune substrate specificity, enhance enzyme stability and

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

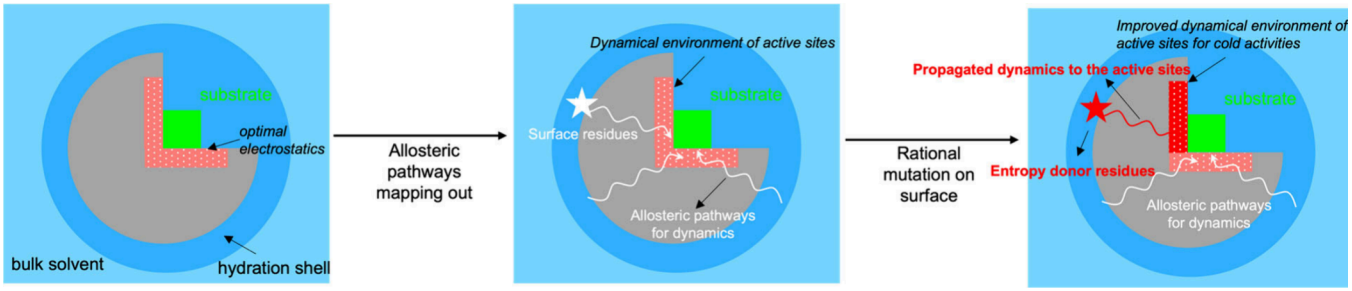


Figure 6. Rational engineering of enzymes for enhanced cold activities. Potential allosteric pathways for dynamics are mapped via computational or experimental tools. A selected allosteric pathway connecting the enzyme surface to the active site is selected(middle) and the surface residues are mutated into entropy donor residues(right). The entropy donor residues will then propagate dynamics into the enzyme core and improve its dynamical environment for enhanced cold activities(right).

promote promiscuity.[92] Detailed examples have been summarized by Kreβ et al.[92]

As demonstrated in the study of adenylate kinase, the dynamics initiated on the surface through the disruption of the secondary structures can be separately propagated to affect both substrate binding and active site catalysis. A protein is generally treated as a domino-like sequential set of amino acids in which allostery is described as the spatial interactions between residues. The allosteric pathway identification methods can be roughly categorized as either computational or experimental.[42] The computational methods include a physics-based approach, an evolutionary approach, α-value analysis and double mutant cycles, while the experimental methods includes NMR, small molecule screening, and crystallography.[42] Computational techniques such as dynamical network analysis and hybrid methods to identify the allosteric pathways have been summarized and their strengths were also compared.[93] Among computational techniques, B-factors have also been employed to study the dynamic allosteric effect in Integrin and generate robust enzymes from cold-adapted lipase C.[94] An excellent review by Sun et al. on B-factors utility in protein science to interpret rigidity, flexibility and internal motion has been published.[95] Recent advancements in network theories and evolutionary analysis have been reviewed and their applications in allosteric regulation have been discussed.[96] Chatzigoulas et al. also summarized the computational tools and databases for identifying and characterizing allosteric pathways.[97] Novel strategies to predict allosteric communication were also reviewed.[98−100] It is worth noting that one appealing tool called "Ohm" was built based on a network modeling approach which can yield solutions more efficiently than simulation-based modeling.[101] A computational tool for statistical coupling analysis (SCA) has also proven successful in guiding enzyme modification.[102,103] This approach assumes that evolutionary pressure preserved allosteric pathways in proteins. It requires the availability of enzyme family members and uses multiple sequence alignment (MSA). Ranganathan et al. proposed that the covaried residues are important for the evolved functions of the enzymes, and the coupling energy is calculated based on the probability of change of one residue *i* related to a change in other residue *j*.[104] SCA was also successfully applied in our study to modify the temperature adaptation of bovine trypsin (unpublished). In this work, the coupling residues of bovine trypsin were classified into 3 sectors across the whole enzyme structure. Sector residues that are at least 12 Å away from the catalytic triad were replaced by the most frequently used residues in the psychrophilic salmon trypsin family. The resulting mutants all showed at least 5 fold higher specific activity across a wide temperature range. In addition, the optimal temperatures of two such mutants were reduced by 15 and 10 degrees, respectively, compared with the wild type. To summarize, the utilization of both experimental and computational tools to predict the dynamic allosteric pathway can be a feasible approach to rationally design and engineer improved cold enzymatic activities.

CONCLUSION

In the context of carbon neutrality and green manufacturing, engineering enzymes for cold activity has attracted much attention in the past several decades (Figure 6).[105] Low temperatures tend to slow the kinetics necessary for catalysis and thus reduce the enzyme activity. Therefore, how to improve the catalytic kinetics at low temperatures is the core question for rational engineering cold activity. Different theories have been advanced to understand the underlying rules governing enzyme temperature adaptation. Active site flexibility theory suggests an enzyme can become more active at lower temperatures by simply introducing flexibility into the active sites to increase the dynamic motion necessary for catalysis. However, engineering the protein core can be harmful and easily deactivate the enzyme activity. Protein surface softness theory suggests that enzyme temperature adaptation is determined by the protein surface where more constraints on the surface motion will modify a psychrophilic enzyme into a mesophilic one. But only selected surface residue changes resulted in cold activity improvement, which hints that key residues on the protein surface are playing an important role in enzyme temperature adaptation. Both of these two theories shed light on enzyme temperature adaptation but cannot guarantee the success for enzyme temperature modification.

The thesis of the dynamical allostery model is that the enzyme surface and active site dynamics are connected so that these allosteric pathway can be manipulated to change catalytic efficiency. Compared with the active site flexibility and surface softness theories, the allosteric dynamic theory is more reasonable as it can explain both of the previous theories and also provide a feasible approach to rationally engineer improved cold enzyme activity. The availability of both experimental and computational tools provides methods for mapping out allosteric pathways for rational cold activity engineering. We suggest that future applications of these approaches are likely to improve predictivity while also proving more use in enzymes.

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528

AUTHOR INFORMATION

Corresponding Author

James Swartz − *Department of Chemical Engineering and Departmnt of Bioengineering, Stanford University, Palo Alto, California 94305, United States;* orcid.org/0000-0002-0711-8437; Email: jswartz@stanford.edu

Author

Weigao Wang − *Department of Chemical Engineering, Stanford University, Palo Alto, California 94305, United States;* orcid.org/0000-0003-2140-2044

Complete contact information is available at: https://pubs.acs.org/10.1021/acscatal.4c02580

Author Contributions

W.W.: conceptualization, investigation, writing−original draft, writing−review and editing. J.S.: writing−review and editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Mark Blenner for initial editing. The authors thank the following individuals for helpful comments and edits: Molly Wintenberg, Dyllan Rives, and Sivachandiran Somu. The authors thank Dr. Zhoutong Sun for writing advice.

REFERENCES

(1) From NLM Medline: Sheldon, R. A. Fundamentals of green chemistry: efficiency in reaction design. *Chem. Soc. Rev.* **2012**, *41* (4), 1437−1451.

(2) From NLM Medline: van den Burg, B. Extremophiles as a source for novel enzymes. *Curr. Opin Microbiol* **2003**, *6* (3), 213−218.

(3) Lonhienne, T.; Gerday, C.; Feller, G. Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. *Biochim. Biophys. Acta* **2000**, *1543*, 1−10.

(4) Bruins, M. E.; Janssen, A. E. M.; Boom, R. M. Thermozymes and Their Applications. *Appl. Biochem. Biotechnol.* **2001**, *90*, 155−186.

(5) Demirjian, D. C; Moris-Varas, F.; Cassidy, C. S Enzymes from Extremophiles. *Curr. Opin. Chem. Biol.* **2001**, *5*, 144−151.

(6) Pucci, F.; Rooman, M. Physical and molecular bases of protein thermal stability and cold adaptation. *Curr. Opin Struct Biol.* **2017**, *42*, 117−128.

(7) Paiardini, A.; Gianese, G.; Bossa, F.; Pascarella, S. Structural plasticity of thermophilic serine hydroxymethyltransferases. *Proteins* **2003**, *50* (1), 122−134.

(8) From NLM Medline: Reed, C. J.; Lewis, H.; Trejo, E.; Winston, V.; Evilia, C. Protein adaptations in archaeal extremophiles. *Archaea* **2013**, *2013*, 373275.

(9) Strub, C.; Alies, C.; Lougarre, A.; Ladurantie, C.; Czaplicki, J.; Fournier, D. Mutation of exposed hydrophobic amino acids to arginine to increase protein stability. *BMC Biochem* **2004**, *5*, 9.

(10) Oskarsson, K. R.; Saevarsson, A. F.; Kristjansson, M. M. Thermostabilization of VPR, a kinetically stable cold adapted subtilase, via multiple proline substitutions into surface loops. *Sci. Rep* **2020**, *10* (1), 1045.

(11) Liu, Z.; Lemmonds, S.; Huang, J.; Tyagi, M.; Hong, L.; Jain, N. Entropic contribution to enhanced thermal stability in the thermostable P450 CYP119. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115* (43), No. E10049-E10058.

(12) Karshikoff, A.; Nilsson, L.; Ladenstein, R. Rigidity versus flexibility: the dilemma of understanding protein thermal stability. *FEBS J.* **2015**, *282* (20), 3899−3917.

(13) Cavicchioli, R.; Charlton, T.; Ertan, H.; Mohd Omar, S.; Siddiqui, K. S.; Williams, T. J. Biotechnological uses of enzymes from psychrophiles. *Microb Biotechnol* **2011**, *4* (4), 449−460.

(14) Miyazaki, K.; Wintrode, P. L.; Grayling, R. A.; Rubingh, D. N.; Arnold, F. H. Directed evolution study of temperature adaptation in a psychrophilic enzyme. *J. Mol. Biol.* **2000**, *297* (4), 1015−1026.

(15) Wintrode, P. L.; Miyazaki, K.; Arnold, F. H. Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. *J. Biol. Chem.* **2000**, *275* (41), 31635−31640.

(16) From NLM Medline: Cavicchioli, R.; Siddiqui, K. S.; Andrews, D.; Sowers, K. R. Low-temperature extremophiles and their applications. *Curr. Opin Biotechnol* **2002**, *13* (3), 253−261.

(17) Metpally, R. P.; Reddy, B. V. Comparative proteome analysis of psychrophilic versus mesophilic bacterial species: Insights into the molecular basis of cold adaptation of proteins. *BMC Genomics* **2009**, *10*, 11.

(18) Georlette, D.; Blaise, V.; Collins, T.; D'Amico, S.; Gratia, E.; Hoyoux, A.; Marx, J. C.; Sonan, G.; Feller, G.; Gerday, C. Some like it cold: biocatalysis at low temperatures. *FEMS Microbiol Rev.* **2004**, *28* (1), 25−42.

(19) Åqvist, J.; Isaksen, G. V.; Brandsdal, B. O. Computation of enzyme cold adaptation. *Nature Reviews Chemistry* **2017**, *1* (7), 0051.

(20) From NLM Medline: D'Amico, S.; Marx, J. C.; Gerday, C.; Feller, G. Activity-stability relationships in extremophilic enzymes. *J. Biol. Chem.* **2003**, *278* (10), 7891−7896.

(21) Feller, G. Protein Stability and Enzyme Activity at Extreme Biological Temperatures. *J. Phys.: Condens. Matter* **2010**, *22* (32), 323101.

(22) Sang, P.; Liu, S. Q.; Yang, L. Q. New Insight into Mechanisms of Protein Adaptation to High Temperatures: A Comparative Molecular Dynamics Simulation Study of Thermophilic and Mesophilic Subtilisin-Like Serine Proteases. *Int. J. Mol. Sci.* **2020**, *21* (9), 3128.

(23) From NLM Medline: Khrapunov, S.; Chang, E.; Callender, R. H. Thermodynamic and Structural Adaptation Differences between the Mesophilic and Psychrophilic Lactate Dehydrogenases. *Biochemistry* **2017**, *56* (28), 3587−3595.

(24) From NLM Medline: Altermark, B.; Niiranen, L.; Willassen, N. P.; Smalas, A. O.; Moe, E. Comparative studies of endonuclease I from cold-adapted Vibrio salmonicida and mesophilic Vibrio cholerae. *FEBS J.* **2007**, *274* (1), 252−263.

(25) Sotelo-Mundo, R. R.; et al. The lysozyme from insect (Manduca sexta) is a cold-adaptedenzyme. *Protein Pept Lett.* **2007**, *14* (8), 774−778.

(26) Fields, P. A.; Somero, G. N. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A(4) orthologs of Antarctic notothenioid fishes. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95* (19), 11476−11481.

(27) Russell, R. J.; Gerike, U.; Danson, M. J; Hough, D. W; Taylor, G. L Structural Adaptations of the Cold-Active Citrate Synthase From an Antarctic Bacterium. *Structure* **1998**, *6* (3), 351−361.

(28) Bentahir, M.; Feller, G.; Aittaleb, M.; Lamotte-Brasseur, J.; Himri, T.; Chessa, J.-P.; Gerday, C. Structural, Kinetic, and Calorimetric Characterization of the Cold-Active Phosphoglycerate Kinase From the Antarctic Pseudomonas Sp. TACII18. *J. Biol. Chem.* **2000**, *275* (15), 11147−11153.

(29) Feller, G.; Gerday, C. Psychrophilic enzymes: hot topics in cold adaptation. *Nat. Rev. Microbiol* **2003**, *1* (3), 200−208.

(30) Saavedra, J. M.; Azocar, M. A.; Rodriguez, V.; Ramirez-Sarmiento, C. A.; Andrews, B. A.; Asenjo, J. A.; Parra, L. P. Relevance of Local Flexibility Near the Active Site for Enzymatic Catalysis: Biochemical Characterization and Engineering of Cellulase Cel5A From Bacillus agaradherans. *Biotechnol J.* **2018**, *13* (8), No. e1700669.

(31) Wang, W.; Dasetty, S.; Sarupria, S.; Blenner, M. Rational engineering of low temperature activity in thermoalkalophilic Geobacillus thermocatenulatus lipase. *Biochem. Eng. J.* **2021**, *174*, 108093.

(32) From NLM PubMed-not-MEDLINE: Gault, S.; Jaworek, M. W.; Winter, R.; Cockell, C. S. Perchlorate salts confer psychrophilic characteristics in alpha-chymotrypsin. *Sci. Rep* **2021**, *11* (1), 16523.

(33) Roca, M.; Liu, H.; Messer, B.; Warshel, A. On the Relationship between Thermal Stability and Catalytic Power of Enzymes. *Biochemistry* **2007**, *46*, 15076−15088.
(34) From NLM Medline: Warshel, A. Electrostatic origin of the catalytic power of enzymes and the role of preorganized active sites. *J. Biol. Chem.* **1998**, *273* (42), 27035−27038.
(35) Galperin, M. Y.; Koonin, E. V. Divergence and convergence in enzyme evolution. *J. Biol. Chem.* **2012**, *287* (1), 21−28.
(36) Liu, S.; Ma, J.; Wang, W.; Zhang, M.; Xin, Q.; Peng, S.; Li, R.; Zhu, H. Mutational analysis of highly conserved residues in the phage phiC31 integrase reveals key amino acids necessary for the DNA recombination. *PLoS One* **2010**, *5* (1), No. e8863.
(37) Suemori, A. Conserved and non-conserved residues and their role in the structure and function of p-hydroxybenzoate hydroxylase. *Protein Eng. Des Sel* **2013**, *26* (7), 479−488.
(38) Samanta, M.; Murthy, M. R.; Balaram, H.; Balaram, P. Revisiting the mechanism of the triosephosphate isomerase reaction: the role of the fully conserved glutamic acid 97 residue. *Chembiochem* **2011**, *12* (12), 1886−1896.
(39) Studer, R. A.; Dessailly, B. H.; Orengo, C. A. Residue mutations and their impact on protein structure and function: detecting beneficial and pathogenic changes. *Biochem. J.* **2013**, *449* (3), 581−594.
(40) Bandyopadhyay, D.; Murthy, M. R.; Balaram, H.; Balaram, P. Probing the role of highly conserved residues in triosephosphate isomerase-analysis of site specific mutants at positions 64 and 75 in the Plasmodial enzyme. *FEBS J.* **2015**, *282* (20), 3863−3882.
(41) Wang, W.; Dasetty, S.; Sarupria, S.; Blenner, M. Rational engineering of low temperature activity in thermoalkalophilic Geobacillus thermocatenulatus lipase. *Biochemical Engineering Journal* **2021**, *174*, 108093.
(42) Dokholyan, N. V. Controlling Allosteric Networks in Proteins. *Chem. Rev.* **2016**, *116* (11), 6463−6487.
(43) Lu, S.; Li, S.; Zhang, J. Harnessing allostery: a novel approach to drug discovery. *Med. Res. Rev.* **2014**, *34* (6), 1242−1285.
(44) Bjelic, S.; Brandsdal, B. O.; Aqvist, J. Cold adaptation of enzyme reaction rates. *Biochemistry* **2008**, *47* (38), 10049−10057.
(45) Isaksen, G. V.; Aqvist, J.; Brandsdal, B. O. Protein surface softness is the origin of enzyme cold-adaptation of trypsin. *PLoS Comput. Biol.* **2014**, *10* (8), No. e1003813.
(46) Isaksen, G. V.; Aqvist, J.; Brandsdal, B. O. Enzyme surface rigidity tunes the temperature dependence of catalytic rates. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (28), 7822−7827.
(47) Åqvist, J.; Isaksen, G. V.; Brandsdal, B. O. Computation of enzyme cold adaptation. *Nat. Rev. Chem.* **2017**, *1* (7), 0051.
(48) Aqvist, J.; Kazemi, M.; Isaksen, G. V.; Brandsdal, B. O. Entropy and Enzyme Catalysis. *Acc. Chem. Res.* **2017**, *50* (2), 199−207.
(49) Socan, J.; Kazemi, M.; Isaksen, G. V.; Brandsdal, B. O.; Aqvist, J. Catalytic Adaptation of Psychrophilic Elastase. *Biochemistry* **2018**, *57* (20), 2984−2993.
(50) Socan, J.; Isaksen, G. V.; Brandsdal, B. O.; Aqvist, J. Towards Rational Computational Engineering of Psychrophilic Enzymes. *Sci. Rep* **2019**, *9* (1), 19147.
(51) From NLM Medline: Koenekoop, L.; van der Ent, F.; Purg, M.; Aqvist, J. The Activation Parameters of a Cold-Adapted Short Chain Dehydrogenase Are Insensitive to Enzyme Oligomerization. *Biochemistry* **2022**, *61* (7), 514−522.
(52) From NLM Medline: Noby, N.; Auhim, H. S.; Winter, S.; Worthy, H. L.; Embaby, A. M.; Saeed, H.; Hussein, A.; Pudney, C. R.; Rizkallah, P. J.; Wells, S. A.; et al. Structure and in silico simulations of a cold-active esterase reveals its prime cold-adaptation mechanism. *Open Biol.* **2021**, *11* (12), 210182.
(53) Kragelund, B. B.; Poulsen, K.; Andersen, K. V.; Baldursson, T.; Krøll, J. B.; Neergard, T. B.; Jepsen, J.; Roepstorff, P.; Kristiansen, K.; Poulsen, F. M.; Knudsen, J. Conserved Residues and Their Role in the Structure, Function, and Stability of Acyl Coenzyme A Binding Protein. *Biochemistry* **1999**, *38*, 2386−2394.
(54) From NLM Medline: Narayanan, C.; Gagne, D.; Reynolds, K. A.; Doucet, N. Conserved amino acid networks modulate discrete functional properties in an enzyme superfamily. *Sci. Rep* **2017**, *7* (1), 3207.
(55) Ramanathan, A.; Agarwal, P. K. Evolutionarily conserved linkage between enzyme fold, flexibility, and catalysis. *PLoS Biol.* **2011**, *9* (11), No. e1001193.
(56) Allain, A.; Chauvot de Beauchene, I.; Langenfeld, F.; Guarracino, Y.; Laine, E.; Tchertanov, L. Allosteric pathway identification through network analysis: from molecular dynamics simulations to interactive 2D and 3D graphs. *Faraday Discuss.* **2014**, *169*, 303−321.
(57) Halabi, N.; Rivoire, O.; Leibler, S.; Ranganathan, R. Protein sectors: evolutionary units of three-dimensional structure. *Cell* **2009**, *138* (4), 774−786.
(58) Ranganathan, R.; Lu, K. P.; Hunter, T.; Noel, J. P. Structural and Functional Analysis of the Mitotic Rotamase Pin1 Suggests Substrate Recognition Is Phosphorylation dependent. *Cell* **1997**, *89*, 875−886.
(59) Agarwal, P. K.; Geist, A.; Gorin, A. Protein Dynamics and Enzymatic Catalysis: Investigating the Peptidyl-Prolyl Cis-Trans Isomerization Activity of Cyclophilin A. *Biochemistry* **2004**, *43* (33), 10605.
(60) Agarwal, P. K. Role of Protein Dynamics in Reaction Rate Ehnancement by Enzymes. *J. Am. Chem. Soc.* **2005**, *127*, 15248−15256.
(61) From NLM PubMed-not-MEDLINE: D'Amico, R. N.; Bosken, Y. K.; O'Rourke, K. F.; Murray, A. M.; Admasu, W.; Chang, C. A.; Boehr, D. D. Substitution of a Surface-Exposed Residue Involved in an Allosteric Network Enhances Tryptophan Synthase Function in Cells. *Front Mol. Biosci* **2021**, *8*, 679915.
(62) From NLM Medline: Agarwal, P. K.; Billeter, S. R.; Rajagopalan, P. T.; Benkovic, S. J.; Hammes-Schiffer, S. Network of coupled promoting motions in enzyme catalysis. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (5), 2794−2799.
(63) From NLM Medline: Wang, L.; Goodey, N. M.; Benkovic, S. J.; Kohen, A. Coordinated effects of distal mutations on environmentally coupled tunneling in dihydrofolate reductase. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (43), 15753−15758.
(64) From NLM Medline: Naganathan, A. N. Modulation of allosteric coupling by mutations: from protein dynamics and packing to altered native ensembles and function. *Curr. Opin Struct Biol.* **2019**, *54*, 1−9.
(65) Liao, M. L.; Somero, G. N.; Dong, Y. W. Comparing mutagenesis and simulations as tools for identifying functionally important sequence changes for protein thermal adaptation. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (2), 679−688.
(66) From NLM Medline: Galdadas, I.; Qu, S.; Oliveira, A. S. F.; Olehnovics, E.; Mack, A. R.; Mojica, M. F.; Agarwal, P. K.; Tooke, C. L.; Gervasio, F. L.; Spencer, J. Allosteric communication in class A beta-lactamases occurs via cooperative coupling of loop dynamics. *Elife* **2021**, *10*, 1 DOI: 10.7554/eLife.66567.
(67) Agarwal, P. K. A Biophysical Perspective on Enzyme Catalysis. *Biochemistry* **2019**, *58* (6), 438−449 From NLM Medline.
(68) Dong, Y. W.; Liao, M. L.; Meng, X. L.; Somero, G. N. Structural flexibility and protein adaptation to temperature: Molecular dynamics analysis of malate dehydrogenases of marine molluscs. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115* (6), 1274−1279.
(69) Saavedra, H. G.; Wrabl, J. O.; Anderson, J. A.; Li, J.; Hilser, V. J. Dynamic allostery can drive cold adaptation in enzymes. *Nature* **2018**, *558* (7709), 324−328.
(70) From NLM Medline: Socan, J.; Purg, M.; Aqvist, J. Computer simulations explain the anomalous temperature optimum in a cold-adapted enzyme. *Nat. Commun.* **2020**, *11* (1), 2644.
(71) From NLM Medline: Hacisuleyman, A.; Erman, B. Entropy Transfer between Residue Pairs and Allostery in Proteins: Quantifying Allosteric Communication in Ubiquitin. *PLoS Comput. Biol.* **2017**, *13* (1), No. e1005319.
(72) Briggs, J. M.; Hacisuleyman, A.; Erman, B. Entropy Transfer between Residue Pairs and Allostery in Proteins: Quantifying

Allosteric Communication in Ubiquitin. *PLOS Computational Biology* **2017**, *13* (1), 1.
(73) Keul, N. D.; Oruganty, K.; Schaper Bergman, E. T.; Beattie, N. R.; McDonald, W. E.; Kadirvelraj, R.; Gross, M. L.; Phillips, R. S.; Harvey, S. C.; Wood, Z. A. The entropic force generated by intrinsically disordered segments tunes protein function. *Nature* **2018**, *563* (7732), 584−588.
(74) Barman, A.; Hamelberg, D. Coupled Dynamics and Entropic Contribution to the Allosteric Mechanism of Pin1. *J. Phys. Chem. B* **2016**, *120* (33), 8405−8415.
(75) Kalescky, R.; Zhou, H.; Liu, J.; Tao, P. Rigid Residue Scan Simulations Systematically Reveal Residue Entropic Roles in Protein Allostery. *PLoS Comput. Biol.* **2016**, *12* (4), No. e1004893.
(76) La Sala, G.; Decherchi, S.; De Vivo, M.; Rocchia, W. Allosteric Communication Networks in Proteins Revealed through Pocket Crosstalk Analysis. *ACS Cent Sci.* **2017**, *3* (9), 949−960.
(77) From NLM Medline: Hong, N. S.; Petrovic, D.; Lee, R.; Gryn'ova, G.; Purg, M.; Saunders, J.; Bauer, P.; Carr, P. D.; Lin, C. Y.; Mabbitt, P. D.; et al. The evolution of multiple active site configurations in a designed enzyme. *Nat. Commun.* **2018**, *9* (1), 3900.
(78) From NLM Medline: Wolf-Watz, M.; Thai, V.; Henzler-Wildman, K.; Hadjipavlou, G.; Eisenmesser, E. Z.; Kern, D. Linkage between dynamics and catalysis in a thermophilic-mesophilic enzyme pair. *Nat. Struct Mol. Biol.* **2004**, *11* (10), 945−949.
(79) Muller, C.; Schlauderer, G.; Reinstein, J; Schulz, G. Adenylate kinase motions during catalysis: an energetic counterweight balancing substrate binding. *Structure* **1996**, *4* (2), 147.
(80) Christoph, W.; Muller, G. E. S. Structure of the Complex Between Adenylate Kinase from Escherichia coli and the Inhibitor ApSA Refined at 1.9 Angstrom Resolution. *J. Mol. Biol.* **1992**, *224*, 19.
(81) From NLM Medline: Bhabha, G.; Lee, J.; Ekiert, D. C.; Gam, J.; Wilson, I. A.; Dyson, H. J.; Benkovic, S. J.; Wright, P. E. A dynamic knockout reveals that conformational fluctuations influence the chemical step of enzyme catalysis. *Science* **2011**, *332* (6026), 234−238.
(82) From NLM Medline: Boekelheide, N.; Salomon-Ferrer, R.; Miller, T. F. 3rd. Dynamics and dissipation in enzyme catalysis. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (39), 16159−16163.
(83) From NLM Medline: Kohen, A. Role of dynamics in enzyme catalysis: substantial versus semantic controversies. *Acc. Chem. Res.* **2015**, *48* (2), 466−473.
(84) From NLM PubMed-not-MEDLINE: Bolhuis, P. G.; Chandler, D.; Dellago, C.; Geissler, P. L. Transition path sampling: throwing ropes over rough mountain passes, in the dark. *Annu. Rev. Phys. Chem.* **2002**, *53*, 291−318.
(85) From NLM Medline: Gobeil, S. M. C.; Clouthier, C. M.; Park, J.; Gagne, D.; Berghuis, A. M.; Doucet, N.; Pelletier, J. N. Maintenance of native-like protein dynamics may not be required for engineering functional proteins. *Chem. Biol.* **2014**, *21* (10), 1330−1340.
(86) From NLM Medline: Tokuriki, N.; Jackson, C. J. Enzyme dynamics and engineering: one step at a time. *Chem. Biol.* **2014**, *21* (10), 1259−1260.
(87) From NLM Medline: Warshel, A.; Bora, R. P. Perspective: Defining and quantifying the role of dynamics in enzyme catalysis. *J. Chem. Phys.* **2016**, *144* (18), 180901.
(88) From NLM Medline: Pisliakov, A. V.; Cao, J.; Kamerlin, S. C.; Warshel, A. Enzyme millisecond conformational dynamics do not catalyze the chemical step. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (41), 17359−17364.
(89) From NLM Medline: Kamerlin, S. C.; Warshel, A. At the dawn of the 21st century: Is dynamics the missing link for understanding enzyme catalysis? *Proteins* **2010**, *78* (6), 1339−1375.
(90) From NLM Medline: McGeagh, J. D.; Ranaghan, K. E.; Mulholland, A. J. Protein dynamics and enzyme catalysis: insights from simulations. *Biochim. Biophys. Acta* **2011**, *1814* (8), 1077−1092.
(91) Roca, L.; Hanhart, C.; Oset, E.; Meißner, U.-G. Testing the natue of the Lambda(1520)-resonance in proton-induced production. *Eur. Phys. J. A* **2006**, *27*, 373−380.
(92) From NLM Medline: Kress, N.; Halder, J. M.; Rapp, L. R.; Hauer, B. Unlocked potential of dynamic elements in protein structures: channels and loops. *Curr. Opin Chem. Biol.* **2018**, *47*, 109−116.
(93) From NLM Medline: Feher, V. A.; Durrant, J. D.; Van Wart, A. T.; Amaro, R. E. Computational approaches to mapping allosteric pathways. *Curr. Opin Struct Biol.* **2014**, *25*, 98−103.
(94) Cesarini, S.; Bofill, C.; Pastor, F. I. J.; Reetz, M. T.; Diaz, P. A thermostable variant of P. aeruginosa cold-adapted LipC obtained by rational design and saturation mutagenesis. *Process Biochem.* **2012**, *47* (12), 2064−2071.
(95) From NLM Medline: Sun, Z.; Liu, Q.; Qu, G.; Feng, Y.; Reetz, M. T. Utility of B-Factors in Protein Science: Interpreting Rigidity, Flexibility, and Internal Motion and Engineering Thermostability. *Chem. Rev.* **2019**, *119* (3), 1626−1665.
(96) From NLM Medline: Liang, Z.; Verkhivker, G. M.; Hu, G. Integration of network models and evolutionary analysis into high-throughput modeling of protein dynamics and allosteric regulation: theory, tools and applications. *Brief Bioinform* **2020**, *21* (3), 815−835.
(97) Chatzigoulas, A.; Cournia, Z. Rational design of allosteric modulators: Challenges and successes. *WIREs Comp. Mol. Sci.* **2021**, *11* (6), e1529.
(98) From NLM Medline: Fan, J.; Liu, Y.; Kong, R.; Ni, D.; Yu, Z.; Lu, S.; Zhang, J. Harnessing Reversed Allosteric Communication: A Novel Strategy for Allosteric Drug Discovery. *J. Med. Chem.* **2021**, *64* (24), 17728−17743.
(99) From NLM Medline: Vishweshwaraiah, Y. L.; Chen, J.; Dokholyan, N. V. Engineering an Allosteric Control of Protein Function. *J. Phys. Chem. B* **2021**, *125* (7), 1806−1814.
(100) From NLM PubMed-not-MEDLINE: East, K. W.; Skeens, E.; Cui, J. Y.; Belato, H. B.; Mitchell, B.; Hsu, R.; Batista, V. S.; Palermo, G.; Lisi, G. P. NMR and computational methods for molecular resolution of allosteric pathways in enzyme complexes. *Biophys Rev.* **2020**, *12* (1), 155−174.
(101) From NLM Medline: Wang, J.; Jain, A.; McDonald, L. R.; Gambogi, C.; Lee, A. L.; Dokholyan, N. V. Mapping allosteric communications within individual proteins. *Nat. Commun.* **2020**, *11* (1), 3862.
(102) From NLM Medline: Tesileanu, T.; Colwell, L. J.; Leibler, S. Protein sectors: statistical coupling analysis versus conservation. *PLoS Comput. Biol.* **2015**, *11* (2), No. e1004091.
(103) From NLM Medline: Liu, M. D.; Warner, E. A.; Morrissey, C. E.; Fick, C. W.; Wu, T. S.; Ornelas, M. Y.; Ochoa, G. V.; Zhang, B. S.; Rathbun, C. M.; Porterfield, W. B.; et al. Statistical Coupling Analysis-Guided Library Design for the Discovery of Mutant Luciferases. *Biochemistry* **2018**, *57* (5), 663−671.
(104) From NLM Medline: Raman, A. S.; White, K. I.; Ranganathan, R. Origins of Allostery and Evolvability in Proteins: A Case Study. *Cell* **2016**, *166* (2), 468−480.
(105) Chen, Q.; Wu, Y.; Huang, Z.; Zhang, W.; Chen, J.; Mu, W. Cold-active enzymes in the dairy industry: Insight into cold adaption mechanisms and their applications. *Trends in Food Science & Technology* **2022**, *125*, 126−135.

https://doi.org/10.1021/acscatal.4c02580
ACS Catal. 2024, 14, 12518−12528