How enzymes adapt: lessons from directed evolution

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Enzymes that are adapted to widely different temperature niches are being used to investigate the molecular basis of protein stability and enzyme function. However, natural evolution is complex: random noise, historical accidents and ignorance of the selection pressures at work during adaptation all cloud comparative studies. Here, we review how adaptation in the laboratory by directed evolution can complement studies of natural enzymes in the effort to understand stability and function. Laboratory evolution experiments can attempt to mimic natural evolution and identify different adaptive mechanisms. However, laboratory evolution might make its biggest contribution in explorations of nonnatural functions, by allowing us to distinguish the properties nutured by evolution from those dictated by the laws of physical chemistry.

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In places previously regarded as incapable of sustaining life, such as Antarctic ice fields and volcanic pools, organisms are now being identified for whom these extreme environments are home, sweet home. In most cases, adaptation to such extreme environments has not required completely new molecular machinery; in fact, many 'extremophilic' enzymes are similar to their counterparts from the 'mesophilic' environments we find more hospitable. For example, enzymes that function at very different temperatures can have nearly superimposable three-dimensional structures (Fig. 1). Sequence comparisons indicate that these enzymes are derived from a common ancestral enzyme and have accumulated mutations that allow them to adapt over millions of years.

Exactly how do enzymes acquire this ability to function in such different environments? If we understood these adaptive mechanisms, perhaps we could use this insight to engineer enzymes for other nonnatural conditions. Unfortunately, this understanding has proven elusive because these mechanisms are both many and complex. A folded protein is only slightly more stable than its unfolded counterpart, and the net stability is equivalent to only a few weak interactions out of the hundreds that are present in any given molecule[1]. From ion binding to networks of hydrogen bonds, salt bridges and hydrophobic interactions, each interaction involved in protein stability has its own, complex temperature dependence. Our understanding of these interactions is incomplete, and we cannot reliably predict how these interactions combine to yield a more or less stable enzyme. Similar weak interactions underlie ligand binding and catalysis; however, the molecular basis of function is even more elusive.

We have large numbers of related molecules that are well adapted to these different environments. Why haven't we been able to use these molecules to uncover the rules governing environment-driven protein evolution? With respect to the problem of thermal adaptation, comparative studies of related enzymes from mesophilic, thermophilic and psychrophilic organisms point to many interesting differences in sequence, structure, function, dynamics, and thermodynamic properties[2–4]. However, we encounter several problems in interpreting these differences and assigning specific sequence changes to particular enzyme behaviors.

Natural sequences are separated by large distances

A thermophilic enzyme might share only 20–30% of its amino acid sequence with its counterparts that have adapted to cooler climates possibly leaving hundreds of differences among a typical set of sequences[5] (Fig. 1). Which of these differences are central to the process of thermal adaptation and which are mere side effects (e.g. the results of neutral drift or even adaptation to other selective pressures)? The large number of differences between two sequences presents a serious hurdle for anyone attempting to relate sequence variation to specific functional consequences. To test all the amino acid substitutions and their combinations is clearly impossible, and such large evolutionary distances make it very difficult to uncover any general rules, including rules that could be used for stabilizing other less-stable proteins.

What evolutionary pressures led to today's enzymes?

Another serious difficulty facing comparative studies is the identification of which enzyme properties have evolved under selective pressure. For example, did the ancestoral enzyme live at high temperature and today's psychrophilic enzyme adapt for catalysis in the cold? Or did an ancient cold-active enzyme have to learn how to function at high temperature? These scenarios will generate very different molecular solutions and diverse interpretations of functional differences.

In addition, organisms are subject to complex combinations of selective pressures. For example, extremophiles from deep-sea hydrothermal vents must adapt to both high temperature and high pressure, whereas bacteria and archaea found in hot springs might have adapted to high temperature and high acidity. Determining which sequence and structural differences reflect adaptation to which selective pressure presents a daunting task in itself.

Furthermore, not all differences in enzyme properties reflect adaptation. For example, neutral mutations are

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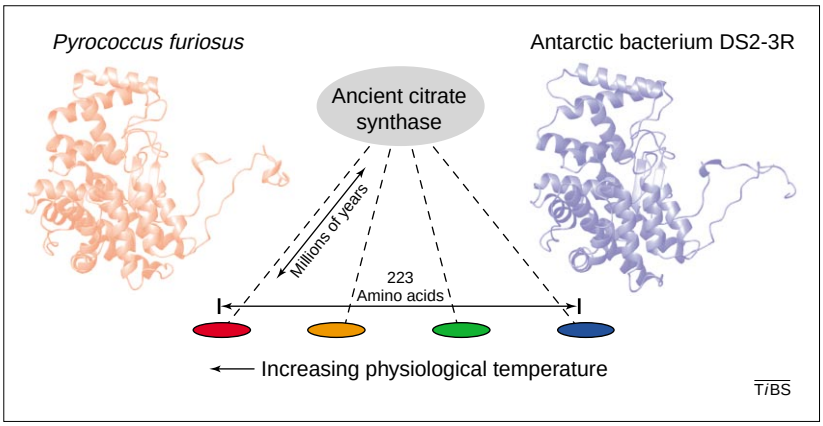

Fig. 1. Divergent evolution leads to adaptation to different environments. Although evolutionary distances can be large (today's enzymes can differ at hundreds of amino acid positions), three-dimensional structures remain very similar. This is illustrated here for a subunit of citrate synthase isolated from a thermophilic (red) and a psychrophilic (blue) organism, which differs at 223 amino acid positions between the two organisms[3,4]. Today's citrate synthases (depicted by colored ovals) from organisms living over a range of temperatures are all derived from an ancient enzyme of the same structure. However, much evolution is either neutral or nearly neutral, and many sequence changes probably only lead to small, if any, changes in functional properties. This can confound comparative studies that aim to uncover adaptive mechanisms.

neutral with respect to fitness but not necessarily to all enzyme behaviors. Mutations that are deleterious to a property that is not subject to natural selection can accumulate and the property will probably decline; however, the process is random and contains little information that can be used to elucidate mechanisms[6].

Biological relevance versus physical chemistry

To elucidate adaptive mechanisms, we must also distinguish between the enzyme behaviors that are dictated by biology and those that are dictated by the laws of physical chemistry. Biological relevance is a further constraint placed on proteins above and beyond any need to satisfy the laws of physics. Biology, and specifically evolution, should not be neglected when we attempt to explain enzyme function.

A good example is the apparent trade-off between thermostability and catalytic activity in enzymes that have evolved to suit different temperature niches (Fig. 2). The explanation most commonly offered for this often-observed trade-off is that, during the course of evolution, enzymes have adjusted the strength and number of their stabilizing interactions to optimize the balance between rigidity (for stability) and flexibility (for activity) at their physiologically relevant temperatures[7,8]. Hence, to achieve catalytic efficiency comparable to that of a mesophilic enzyme at its natural physiological temperature, a psychrophilic enzyme functioning at 10°C must exhibit thermal motions of the same magnitude as those of the mesophilic enzyme functioning at 37°C. However, when the cold-adapted enzyme is exposed to mesophilic temperatures, these motions become so great that they can lead to the loss of native structure. Conversely, when the mesophilic enzyme is cooled to 10°C, the reduced thermal fluctuations will diminish the conformational mobility and consequently compromise catalytic efficiency.

This argument provides an appealing physical–chemical explanation for the stability and activity behaviors of homologous enzymes that have adapted to different temperatures (Fig. 2). However, all these enzymes are the products of evolution and although they are certainly subject to the laws of physics and chemistry, the evolutionary process imposes its own constraints. Evolution produces the special subset of functional enzymes that are biologically relevant and can be generated from extant enzymes by mutation and selection.

Laboratory evolution of enzymes

Experiments that enable us to evolve enzymes in the laboratory under controlled conditions and well-defined selection pressures can help us clarify the confusion introduced by natural evolution. First, we can generate functional changes of the enzymes with only minimal changes in sequence. For example, we can determine the number of amino acid substitutions taken to evolve a thermophilic enzyme in the laboratory. Because the mutations are all, or nearly all, adaptive, the mechanisms of adaptation will be easier to discern. Second, we can generate different evolutionary scenarios and monitor the adaptation process, with access to all intermediates. To the extent that this mimics nature, we could even try to retrace history. Moreover, we can determine whether the mutations parallel those that are found in natural homologous enzymes or whether there are multiple

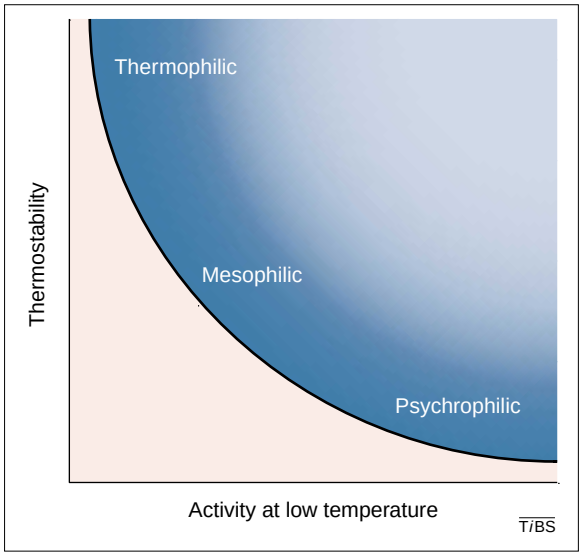

Fig. 2. Homologous enzymes adapted to different temperatures show a trade-off between catalytic activity at low temperatures (high for enzymes from psychrophilic organisms but generally low for enzymes from thermophiles) and thermostability (high for thermophilic enzymes but low for enzymes from psychrophiles). These naturally occurring enzymes lie in the darker shaded area of the figure, bounded on one side (pink area) by the minimal stability and activity that are required for biological function, and on the other side (lighter shaded area in top, right-hand quadrant) by enzymes that are both highly thermostable and highly active at low temperature, which are generally not found in nature. However, laboratory experiments demonstrate that this region of the function space is accessible to evolution, and so we can conclude that active, stable enzymes are physically possible, but not biologically relevant.

pathways that lead to the same functional result. It might even be possible to shed light on the properties of the ancestral molecules and on the pathways taken in natural evolution. Third, with laboratory evolution, apart from the requirement that the enzyme be functionally expressed in the host organism, we are free of biological constraints, and free to access all physically possible enzymes that the search algorithm (mutation, recombination and screening/selection) can generate. Furthermore, we can also attempt to create enzymes for which there are no natural counterparts. For example, can we make enzymes that are both thermostable and highly active at low temperature? If so, we must look beyond the appealing physical explanation for the apparent incompatibility of these two properties in natural enzymes.

Directed evolution techniques

The basic algorithm of directed evolution mimics, in some ways, that of natural evolution. The two key steps are generating molecular diversity and identifying the improved variants. The most widely used approaches to generating diversity are random point mutagenesis and *in vitro* recombination. For random mutagenesis, an error-prone version of the polymerase chain reaction (PCR) is particularly convenient[9]. Under the correct conditions (e.g. divalent cations and pH), the polymerase will misincorporate bases at a controllable rate during gene amplification. A low error rate (2–3 base substitutions or ~1 amino acid substitution per sequence per generation) accumulates mostly adaptive mutations, whereas higher error rates generate neutral and deleterious mutations, which confound analysis. Beneficial mutations in multiple variants can be combined using recombination methods such as Stemmer's DNA shuffling[10] or StEP (staggered extension process)[11]. Recombination also removes neutral and deleterious mutations.

The next step is to identify the enzyme variants that have improvements in the desired properties. In this sense, directed evolution is more like breeding than like natural selection. The outcome of the experiment depends crucially on what properties are investigated. Devising screens that are sensitive to the small functional changes that are expected from single amino acid substitutions (e.g. a twofold increase in activity) can be challenging and, because the frequency of improved mutants might only be 1 in 1000, the screen must have low inherent variability.

To investigate the adaptability of a subtilisin protease from a psychrophilic organism[12,13], we used low error rate PCR random mutagenesis and saturation mutagenesis to introduce molecular diversity into the protein and *in vitro* recombination to identify good combinations of beneficial mutations. To screen the mutated enzyme libraries for thermostable variants, we measured the residual activities after incubation at a temperature high enough to largely inactivate the parent enzyme. These values were then compared to the initial activities, and the proteins that survived best were collected (Fig. 3a). Using this method, we could monitor thermostability and activity at low temperature simultaneously (Fig. 3b). A strategy of using iterative cycles of mutagenesis and screening for improved enzymes enabled the accumulation of mutations that were required to adapt to new environments (Fig. 3c).

Selections, in which improvements in enzyme properties are coupled to the survival and growth of the host organism, provide an interesting alternative to screening[14,15]. Selections are powerful tools for laboratory evolution because they allow large numbers of variants (10^6–10^8) to be examined. Unlike screening, selections require that the enzymes exert a biological function. Thus, the selective pressures might mimic those encountered during natural

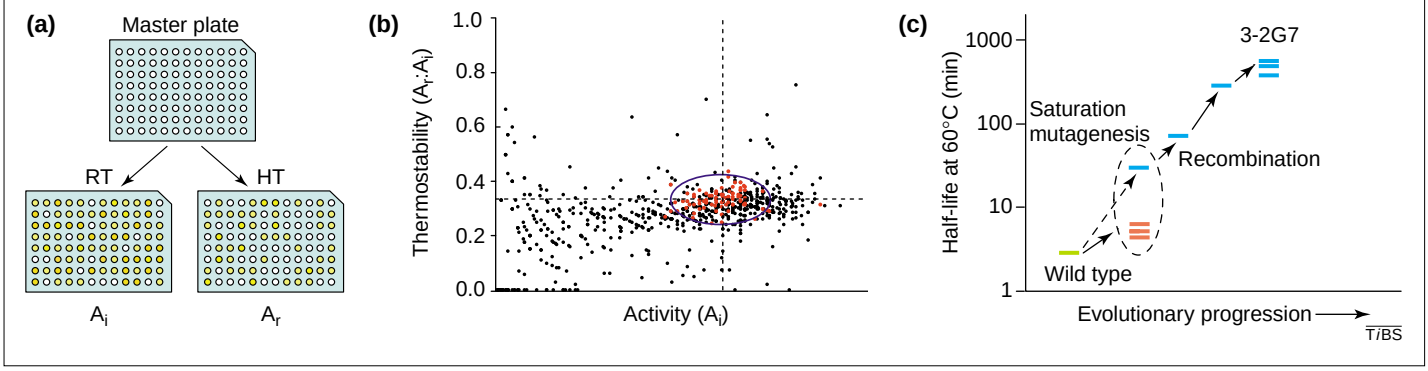

Fig. 3. Directed evolution of a psychrophilic enzyme, subtilisin S41, from the Antarctic bacterium TA41 (Ref. 12). (a) Rapid screening for thermostability and activity is performed by taking replicas from a master plate that contains individual clones. One replica plate is assayed for activity at room temperature (RT), and the second is incubated at high temperature (HT) before activity is measured. The ratio of the residual activity (Ar), calculated from the HT plate, to the initial activity (Ai), from the RT plate, provides a measure of thermostability for this irreversibly inactivated enzyme. (b) Activities and stabilities of random S41 mutants[13]. The distribution of wild-type clones measured under the same conditions (red dots inside ellipse) shows the reproducibility of the screen. Mutants distribute well outside this region. Most improvements in activity come at the cost of stability, and vice versa. (c) Progression of the evolution of S41 thermostability, as measured by the half-life of enzyme activity at 60°C, in 1 mM $CaCl_2$. One of the mutants discovered in the first generation contained two amino acid substitutions in a loop region. These were subjected to saturation mutagenesis, and the best mutant was recombined with other random mutants. Further rounds of random mutagenesis and screening produced 3-2G7, which has seven amino acid substitutions.

evolution more closely. However, selections suffer from serious drawbacks; in particular, the evolving enzyme must contribute to a growth advantage for the cell, which limits the range of enzymes and properties that we can study. In addition, selections are inappropriate if the goal is to explore properties, or combinations of properties, that are not biologically relevant.

Temperature adaptation in the laboratory

Through directed evolution studies, we have learned that enzymes can adapt rapidly to new environments and traverse a wide range of temperatures. Highly thermostable variants of mesophilic[14,16–18], and even psychrophilic[12,13], enzymes have been developed. These laboratory-evolved enzymes are equivalent in stability to many naturally occurring thermophilic enzymes and, similar to their natural counterparts, are also more resistant to proteolytic digestion and chemical denaturation. Laboratory evolution has also generated cold-active variants of mesophilic and thermophilic enzymes[15,19–21].

Evolution of thermostability

Laboratory evolution can generate large increases in thermostability with very few amino acid substitutions. In one study, we converted a psychrophilic enzyme, subtilisin S41 from Antarctic *Bacillus* TA41, into its thermophilic counterpart[12] (Fig. 3). At 60°C, variant 3-2G7 has a half-life of 449 min, which is more than 500 times that of the wild-type enzyme[12] and considerably more than its mesophilic homologs, subtilisin BPN′ and SSII, whose half-lives under the same conditions are only 28 and 34 min, respectively. Melting curves measured by circular dichroism show an increase in stability of ~23°C, yet this 5th-generation variant differs at only seven amino acids from wild-type S41 (i.e. the two enzymes exhibit 97.7% identity). For comparison, subtilisin S41 differs from its natural thermophilic homolog thermitase at 178 positions (35% identity), not counting various gaps and insertions.

Because subtilisin-like proteases are found in organisms that are adapted to a wide range of environments, this family of enzymes provides an opportunity to compare laboratory adaptation mechanisms with natural evolution. S41 is highly active at low temperatures, but unstable compared with its mesophilic relatives. A striking feature of S41 is its extended loops, which are not found in the common mesophilic subtilisins such as BPN′. Another notable feature of the psychrophilic enzyme is its high content of surface Asp residues, which number 22. Both of these features have been suggested to impart the special properties of the cold-adapted enzyme[7]. Looking through the sequence databases for close relatives of S41, we found that the mesophilic subtilisin SSII from *Bacillus sphaericus* is very similar in sequence to S41. SSII also has the same extended loops as does S41[12,22], and 19 Asp residues, most of which correspond to those in S41. However, SSII has the stability of a mesophilic enzyme. Thus, neither the existence of the extended loops nor the high Asp content can explain the special behavior of the cold-adapted S41.

During the laboratory evolution process, did we rediscover sequence changes already present in SSII and other subtilisins more thermostable than S41, or do the mutations represent new solutions to making more stable subtilisins? Although two of the mutations are found in the closely related SSII enzyme, the other five are not in SSII or mesophilic subtilisins BPN′, E and Carlsberg. It is probably true that natural evolution has not explored all of the available solutions to the thermostability challenge. However, it is also true that the effects of specific mutations depend on their context, so a mutation that is stabilizing in S41 might not necessarily be so in another sequence. Most importantly, we must remember that natural selection chooses the fittest organism, and so we are left with the challenge of identifying the enzyme properties that contribute to this fitness. We do not know whether the low relative stability of S41 is even biologically meaningful.

Stabilization mechanisms

What mechanisms did S41 use to adapt to high temperature in the laboratory? Unfortunately, crystal structures of the wild-type S41 and SSII subtilisins are not yet available. However, data on the stability of S41 in different calcium concentrations, and homology models constructed from the structures of related subtilisins, enabled us to postulate mechanisms by which the psychrophilic enzyme might have become more stable[10]. The introduction of a Pro residue into one of the extended loops (which presumably decreases the entropy of the unfolded state) and increased affinity for calcium ions (also an important stabilizing mechanism for naturally occurring high-temperature subtilisins) both appear to be important for the adaptation of S41 to high temperatures. All seven mutations in the thermostable subtilisin S41 are located on the surface of the protein and, with one exception (Ser145Ile), none occurs in regions of regular secondary structure. Three mutations are found in one of the extended loops and perhaps serve to rigidify this loop. Stabilization mechanisms inferred from molecular modeling include the formation of new salt bridges and improved hydrophobic interactions in the protein interior. None of the mutations is in known calcium binding sites, and so the mechanism for the large increase in calcium affinity remains unknown.

In another directed evolution study, eight rounds of mutagenesis and screening of the mesophilic *p*-nitrobenzyl esterase (pNBE) from *Bacillus subtilis* yielded pNBE variant 8G8, which unfolds at a temperature 17°C higher than does its wild-type progenitor[16,17]. A pair of natural mesophilic and thermophilic esterases might differ at a hundred or more amino acids, whereas wild-type pNBE and 8G8

differ at only 13 amino acids (i.e. less than 3% of their sequence). Crystal structures of both pNBE and its evolved thermostable variant illustrate some notable features of the evolution of this enzyme[23]. One of the most interesting is the fact that two surface loops that are not visible in the wild-type electron density, presumably because of their high mobility, become fixed during the evolution of the thermostable enzyme. The fixing of these loops creates ten new hydrogen bonds. These changes are mediated by long-range interactions; the responsible amino acid substitutions are located outside the loops, underscoring the subtle mechanisms that evolutionary design can recruit. Fixation of the loops happened early in the evolution of these enzymes and prepared a framework for new stabilizing mutations, which included a salt bridge.

The constraining of large-scale loop motions has been a recurring theme in the directed evolution of thermostability. Huimin Zhao evolved the mesophilic subtilisin E into a functional equivalent of its thermophilic homolog thermitase[18]. Molecular dynamics simulations on both wild-type subtilisin E and its laboratory-evolved, thermostable variant 5-3H5 also demonstrated a major role for reducing loop conformational mobility[24]. Constraining loop motions reduced the rate of subtilisin E autolysis as well as the rate of unfolding.

Mechanisms that contribute to the stabilization of pNBE (and other laboratory-evolved enzymes) also include the introduction of new hydrogen bonds and salt bridges, aromatic–aromatic interactions, helix stabilization and changes in side-chain packing. This multitude of stabilization mechanisms mirrors what we know about naturally thermostable enzymes[2] and makes thermostability a relatively easy property to evolve. It also emphasizes how difficult it is to generalize stabilization mechanisms into design rules.

Activities of the evolved enzymes

A second goal of our directed evolution experiments was to test whether it is possible to evolve enzymes that are both thermostable and highly active at low temperature. Therefore, we required that the esterase and the subtilisin protease retain significant activity at room temperature while thermostability increased. We encountered no difficulty in finding thermostable enzymes that retained, and even increased, their activities. In fact, the most thermostable variants are more active than their wild-type progenitors at all temperatures[12,17] (Fig. 4). The specific activity of pNBE 8G8 is at least 3.5 times higher than that of the wild-type enzyme over the whole temperature range, and subtilisin S41 3-2G7 has at least twice the activity of wild-type S41.

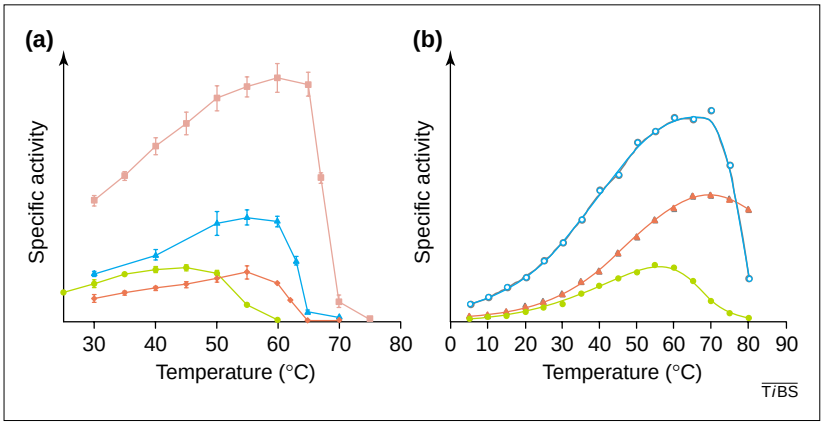

Fig. 4. Temperature dependence of the specific activities (in arbitrary units) of evolved thermophilic enzymes[12,16,17]. (a) *p*-Nitrobenzyl esterase: wild type (green), 4th (red), 6th (cyan) and 8th (pink) generation mutants; (b) subtilisin S41: wild type (green) and 5th generation variant, 3-2G7 (cyan). Activities of mesophilic subtilisin SSII (red) are shown for comparison.

As discussed above, the observation that catalytic activity at moderate and low temperatures decreases with increasing thermostability in naturally occurring enzymes[8,25–27] has led to the claim that there is an inherent trade-off between the rigidity necessary for stability and the flexibility required for enzyme activity. However, thermostable enzymes that retain high activity at low temperature are physically possible; it seems that this behavior does not reflect physical constraints after all, but rather evolutionary constraints. It is possible that natural selection actively avoids highly stable, highly active enzymes. An enzyme that is too stable, for example, might be resistant to degradation within the cell. For thermophilic organisms, high activity at moderate temperatures could translate into catalysts that are so active at elevated physiological temperatures that they hinder cellular function.

Another, very simple evolutionary explanation is that as mutations accumulate during the course of evolution, unconstrained properties will drift (downward). Thus, thermophilic enzymes adapting to cooler environments would lose their high thermal stability, and mesophilic or psychrophilic enzymes adapting to hotter environments could lose their low-temperature activity. This drift would tend to push enzyme function towards the biologically relevant minimum (Fig. 2).

Directed evolution experiments demonstrate how random drift of an unconstrained property affects the evolutionary trajectory[15,19]. Mesophilic subtilisin SSII (Ref. 19) and indoglycerol phosphate synthase[15] were evolved to increase low-temperature activity, with no constraints on enzyme stability other than that required for function. In these experiments, the more active variants did in fact tend to be less thermostable, although this was not always the case. For example, one first-generation SSII variant with a twofold increase in activity was more thermostable than was its wild-type parent[19]. Thus, results from directed evolution[12,14,16,18–20], and site-directed mutagenesis studies[28–31], in addition to the properties of some naturally occurring enzymes[32], demonstrate that there is no inherent physical contradiction that interferes with improving thermostability and low-temperature activity. It is more difficult to make enzymes that are both thermostable and highly active at low temperatures so, without a compelling reason to do so, Nature simply does not.

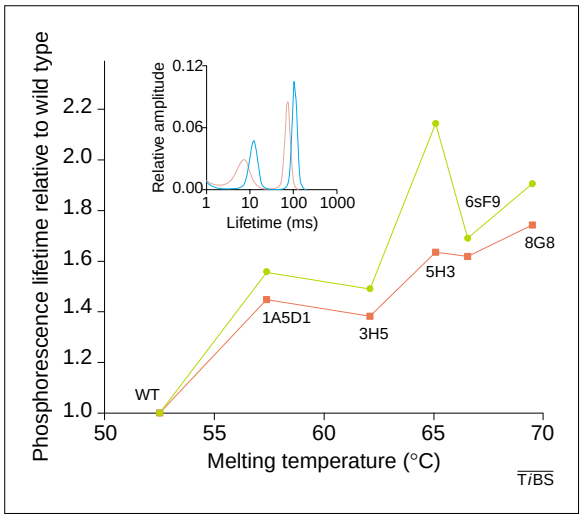

Fig. 5. Room temperature phosphorescence lifetimes for wild-type (WT), *p*NBE and thermostable mutants (described by increases in melting temperature)[17]. The red and green lines represent the short (~10 ms) and long (~100 ms) lifetime components of the phosphorescence, respectively. Data are normalized to wild type. Inset: relative contributions of different lifetimes to the total observed phosphorescence for wild type (pink line) and 8G8 (cyan line). The two major components are clearly visible and, although there is a trend towards longer lifetimes (decreased local flexibility), substantial increases in stability can be accompanied by significant decreases in the phosphorescence lifetimes (indicating greater local flexibility). This is particularly notable in the progression from variant 53H5 to variant 6SF9.

What role does flexibility play in stability and function?

Laboratory evolution has generated sets of closely related enzymes with varying levels of stability and activity. We decided to use these enzymes to try to discern how enzyme flexibility contributes to these behaviors. For this we used Trp phosphorescence to report on the motions of wild-type pNBE and its thermostable variants.[17]. In the absence of oxygen, and near room temperature, Trp phosphorescence is quenched mainly by out-of-plane motions of the indole side chain[33,34]. Constraining the motion of the indole ring in more rigid local environments leads to longer phosphorescence lifetimes. It is possible that differences in local rigidity reflect changes in enzyme thermostability, although they could also arise by random drift alone. However, by measuring lifetimes for the enzymes along the evolved lineage, we could attempt to differentiate between random changes in lifetimes and those that become fixed and are, presumably, related to thermostability.

We found that phosphorescence lifetimes were increased in the first-generation thermostable variant 1A5D1, and that this increase was retained in all of the subsequent generations. The two loops that were not seen in the wild-type structure are already constrained in 1A5D1 (Ref. 23). The same long-range interactions that stabilize the loops probably also restrict the motions of the phosphorescing Trp residues. This widespread loss of conformational entropy is presumably offset by the increase in stability that arises from the burial of hydrophobic residues, introduction of new hydrogen bonds and restriction of solvent accessibility to the protein core as the loop regions become more structured. As stability increased during subsequent generations, we saw a trend towards longer phosphorescence lifetimes. However, sometimes substantial increases in stability were accompanied by decreased lifetimes (Fig. 5). Thus, increased thermostability can coexist with *increases* in local flexibility.

What is the relationship between phosphorescence lifetimes and enzyme activity? We found no correlation between phosphorescence lifetimes and the increased catalytic activities, which could be simply because neither of the phosphorescing Trp residues is located near the active site. It could also mean that the motions affecting phosphorescence lifetimes are irrelevant to activity. It is important to define 'flexibility' when we discuss how this parameter affects other protein properties; locations, amplitudes and timescales of protein motions must all be considered. In addition, catalysis and stability could depend on some motions and be independent of others.

Conclusions

Laboratory evolution is a promising new tool for studying enzyme function and adaptation, which allows us to observe adaptation under controlled conditions. In addition, with entire lineages, and not just the endpoints of evolution, we have a unique vantage point for studying fundamental questions of protein structure and function. Moreover, because we can define the evolutionary pressures, we should be able to explore nonnatural functions to distinguish what is biologically relevant from what is physically possible. We hope that this approach will prove useful for studying protein adaptation in a variety of contexts, both natural and artificial.

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Probing the interface between membrane proteins and membrane lipids by X-ray crystallography

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Biological membranes are composed of a complex mixture of lipids and proteins, and the membrane lipids support several key biophysical functions, in addition to their obvious structural role. Recent results from X-ray crystallography are shedding new light on the precise molecular details of the protein–lipid interface.

Biological membranes are not just inert physical barriers, they are metabolically active and support a wide range of key biochemical processes. These include respiration and photosynthesis, solute transport, motility, cell–cell recognition, signal transduction and protein transport. Those proteins that form an integral part of biological membranes inhabit a complex environment. Over part of their surface they are exposed to the aqueous phase on one or both sides of the membrane, where they interact with water, small hydrophilic ions and molecules, and water-soluble proteins. The remainder of their surface is exposed to the membrane, either the mainly hydrophilic 'interface' region, which forms a layer approximately 15 Å thick on either side of the membrane, or the ~30 Å thick hydrophobic interior of the membrane.

It is widely thought that specific protein–lipid interactions are important for the structural and functional integrity of many key integral membrane proteins from prokaryotic and eukaryotic membranes[1–3]. Specific examples include the plasma membrane Ca^{2+} pump (Ca^{2+}-ATPase)[4], rhodopsin[5], cytochrome *c* oxidase[6,7] and the ADP/ATP carrier from mitochondria[8]. A good match between the hydrophobic protein surface and the surrounding lipids is thought to be important for the stable integration of integral membrane proteins into the lipid bilayer. Interestingly, the introduction of a mismatch through an alteration in the thickness of the hydrophobic core of the membrane, by incorporation of longer or shorter lipids, might provide a mechanism for sorting proteins between different types of membrane in complex membrane systems[9].

X-ray crystallography of membrane proteins

As with soluble proteins, much effort has gone into investigating the structure of membrane proteins by

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