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An appraisal of the enzyme stability-activity trade-off

Scott R. Miller[1,2]

[1]*Division of Biological Sciences, The University of Montana, Missoula, Montana 59812*

[2]*E-mail: scott.miller@umontana.edu*

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A longstanding idea in evolutionary physiology is that an enzyme cannot jointly optimize performance at both high and low temperatures due to a trade-off between stability and activity. Although a stability-activity trade-off has been observed for well-characterized examples, such a trade-off is not imposed by any physical chemical constraint. To better understand the pervasiveness of this trade-off, I investigated the stability-activity relationship for comparative biochemical studies of purified orthologous enzymes identified by a literature search. The nature of this relationship varied greatly among studies. Notably, studies of enzymes with low mean synonymous nucleotide sequence divergence were less likely to exhibit the predicted negative correlation between stability and activity. Similarly, a survey of directed evolution investigations of the stability-activity relationship indicated that these traits are often uncoupled among nearly identical yet phenotypically divergent enzymes. This suggests that the presumptive trade-off often reported for investigations of enzymes with high mean sequence divergence may in some cases instead be a consequence of the degeneration over time of enzyme function in unselected environments, rather than a direct effect of thermal adaptation. The results caution against the general assertion of a stability-activity trade-off during enzyme adaptation.

KEY WORDS: **Adaptation, conditional neutrality, directed evolution, enzyme stability, pleiotropy, protein evolution, trade-off.**

Enzymes are the principal biological catalysts that speed up chemical reactions primarily by lowering the activation energy barrier to converting reactants into products through the stabilization of the transition state (Garcia-Viloca et al. 2004). They are intrinsically flexible, dynamic macromolecules with internal motions that are necessary for the binding of substrates, the release of products and, potentially, the mechanism of catalysis itself (Hammes-Schiffer and Benkovic 2006; Henzler-Wildman et al. 2007). Enzymes are also typically only marginally stable: the properly folded, functionally active ensemble of an enzyme's conformations (i.e., the native state) is only slightly energetically favored over the unfolded state at physiological temperatures (Wintrode and Arnold 2001; Hochachka and Somero 2002). The maintenance of enzyme structure and activity is therefore thought to arise from a delicate balance of stability and flexibility.

Orthologous enzymes from ectotherms adapted to different temperatures often vary in their thermal stabilities, with enzymes from organisms that live in high temperature environments exhibiting greater resistance to unfolding. It is commonly asserted that the mechanism responsible for this enhanced stability is a decrease in flexibility (i.e., increased rigidity) and that, consequently, a more thermostable enzyme is expected to be a slower catalyst than a less stable ortholog at low temperature (Somero 1995; Arnold et al. 2001; Hochachka and Somero 2002; DePristo et al. 2005; Feller 2010). Under this view, natural selection is predicted to compensate for differences in environmental temperature such that an enzyme's binding and catalytic abilities are conserved at the respective physiological temperatures of taxa (Jaenicke and Závodszky 1990; Jaenicke 1991; Somero 1995; Závodszky et al. 1998; Fields 2001).

This presumed trade-off between an enzyme's resistance to unfolding and its ability to perform the conformational changes required for catalysis has been a powerful idea in evolutionary physiology, and there are many reports of a stability-activity trade-off in the literature. Most notably, these include lactate dehydrogenases from fish and marine invertebrates adapted to different temperatures (reviewed by Fields (2001) and Hochachka and Somero (2002)) as well as enzymes from mesophilic versus

extremophilic microorganisms (reviewed by Sterner and Liebel (2001) and Feller (2010)). Despite these examples, however, the generality of a trade-off between enzyme stability and activity remains unclear, and there are both theoretical and empirical reasons to believe that the relationship between these traits may be more complicated.

Thermodynamically, whether an enzyme remains stably folded at a given temperature T is determined by the difference in free energy between its folded and unfolded states (i.e., the free energy of stabilization, ΔG_{stab}), with $\Delta G_{\text{stab}} > 0$ favoring the folded state (Becktel and Schellmann 1987). ΔG_{stab} is a function of the differences between the folded and unfolded states in enthalpy (ΔH^0), entropy (ΔS^0), and heat capacity (ΔC_{p}), respectively:

$$\Delta G_{\text{stab}} = \Delta H^0 - T\Delta S^0 + \Delta C_{\text{p}}\left(T - T^0 - T\ln\left(T/T^0\right)\right), \qquad (1)$$

where T^0 is a reference temperature (Becktel and Schellmann 1987). Stability and activity are only expected to be correlated if increased thermal stability (i.e., an increase in the temperature T_{m} at which $\Delta G = 0$) is conferred by a decrease in enzyme flexibility. Many thermostable enzymes do appear to have lower global flexibility than less stable homologs based on lower rates of molecular motion when measured at the same temperature, for example, by hydrogen/deuterium exchange (Závodszky et al. 1998; Wintrode and Arnold 2001; Karshikoff et al. 2015). Protein stability and rigidity may both increase via an increase in ΔH, such as by the addition of a hydrogen bond (Razvi and Scholtz 2006). For example, the greater stability of the histone from *Methanothermus fervidus*, a hyperthermophile, compared with that from the mesophilic *Methanobacterium formicum* is due to enthalpic changes, including the addition of ion pairs and enhanced hydrophobic interactions in the former (Li et al. 2000).

However, it is evident from equation (1) above that stability may also evolve through a reduction in ΔS and/or ΔC_{p} (Kumar and Nussinov 2001; Sawle and Ghosh 2011), which can produce a more thermostable enzyme without an accompanying decrease in flexibility (Daniel et al. 2003; Karshikoff et al. 2015). In fact, a more thermostable enzyme may be more flexible than a less stable homolog if ΔS is reduced by an increase in the conformational entropy of the native state, whereby there are a greater number of accessible substates without initiation of unfolding compared with a less stable enzyme (Lazaridis et al. 1997; Karshikoff et al. 2015). Indeed, both experimental investigations and molecular dynamics simulation studies of protein motions over a range of time scales have demonstrated that more thermostable proteins are not necessarily less flexible than less stable homologs (Hernandez et al. 2000; Jaenicke 2000; Merkley et al. 2010; Sawle and Ghosh 2011; Karshikoff et al. 2015). For example, rubredoxin from the hyperthermophile *Pyrococcus furiosus* exhibits flexibility comparable to proteins of mesophilic organisms despite an extraordinarily low unfolding rate at 100°C (Hernández et al. 2000). Furthermore, if enzyme flexibility systematically decreased with stability, then one would expect the temperature dependence (Q_{10}) of enzyme catalytic rates to decrease with increasing physiological temperature; however, there is no evidence for a systematic difference in Q_{10} values for enzymes that operate at low, moderate, and high temperatures in nature, respectively (Elias et al. 2014).

Other, historical factors may complicate the relationships among stability, flexibility, and activity. For example, the process of thermodynamic stabilization need not be homogeneous over time. Taking an ancestral protein resurrection approach, Hart et al. (2014) reported that the underlying thermodynamic mechanisms of stabilization have varied among lineages during the evolutionary history of bacterial ribonuclease H1. In addition, aspects of enzyme activity may be subject to other selective pressures besides temperature. Both the enzyme catalytic turnover number (k_{cat}) and the Michaelis constant (K_{m}), a measure of an enzyme's substrate affinity, are often assumed to be negatively correlated with stability based on the proposed mechanism that a more stable and rigid enzyme both binds its substrate(s) more tightly (lower K_{m}) and is a slower catalyst (lower k_{cat}; Somero 1995; Arnold et al. 2001; Hochachka and Somero 2002). However, both may be influenced by selection on the specificity of substrate preference, quantified by the ratio $k_{\text{cat}}/K_{\text{m}}$, as well as the substrate concentration available to an enzyme. This can complicate the inference of the mechanisms responsible for changes in activity, particularly if selection varies among lineages, e.g. along a specialist-generalist axis. In this light, an interesting observation is that ancient reconstructed enzymes appear to be both more thermostable and more promiscuous with respect to substrate utilization than modern enzymes (Risso et al. 2013; Romero-Romero et al. 2016; Trudeau et al. 2016; Wheeler et al. 2016).

Finally, there are many examples of the absence of a stability-activity trade-off among both engineered and natural enzymes. Enzymes engineered to be more thermostable in vitro sometimes evolve without a cost of reduced activity at cold temperature (Arnold et al. 2001). Among natural enzymes, a decoupling of stability and activity has been reported for lactate dehydrogenases of several fish species (Fields and Somero 1997; Holland et al. 1997) and congeneric porcelain crabs (Stillman and Somero 2001), for example. Because a stability-activity trade-off may impose costs for organismal performance at physiological extremes, it has the potential to drive the processes of ecological specialization and niche differentiation along environmental gradients, shape biogeographical patterns, and impact the evolutionary responses of taxa to climate change (Chown et al. 2010; Somero 2010). Addressing the prevalence of the trade-off for enzymes in nature is therefore essential. Here, I tested the prediction of the trade-off mechanism that enzyme stability and activity are

negatively correlated for comparative biochemistry datasets obtained from a comprehensive literature search.

Methods

Identification of natural enzyme datasets

Web of Science searches from 1968 through 2016 were performed under the topic terms (1) "enzyme stability" and "temperature" and (2) "temperature adaptation" and "protein," respectively, with the vast majority of returns from after 1990. To be included in the analysis, data were required for some measure of both (1) enzyme stability (e.g., enzyme denaturation temperature, T_m; enzyme optimal temperature, T_{opt}; time to 50% enzyme inactivation, T_{50}; the free energy of unfolding, ΔG_U) and (2) kinetics assayed at a common garden temperature (e.g., enzyme catalytic turnover number, k_{cat}; the maximal rate of catalysis, V_{max}; enzyme-specific activity; Michaelis constant, K_m).

Phylogenetic comparative analysis

Correlations between stability and activity were estimated by phylogenetic generalized least squares (Martins and Hansen 1997). Where possible, FASTA-formatted nucleotide sequence data were obtained for the sampled enzymes and subsequently aligned with CLUSTALW (Thompson et al. 1994). Maximum likelihood phylogenies were reconstructed with PAUP* version 4.0b10 (Swofford 2002) following selection of a model of sequence evolution using Akaike Information Criterion (AIC) values estimated with Modeltest (Posada and Crandall 1998). Branch length data from phylogenies were incorporated into phylogenetic generalized least squares models implemented with Compare version 4.6 (http://compare.bio.indiana.edu/). For each model, the alpha parameter, which indicates the strength of the constraint on phenotypic evolution, was optimized from the data by REML for values of alpha ranging between zero and 15.5. Alpha specifies how phenotypes of taxa evolve along the phylogeny: a value of zero corresponds to Brownian motion, whereas, for positive values, phenotypes evolve by an Ornstein-Uhlenbeck "rubber-band" process. Brownian motion captures the linear decrease in the phenotypic covariance of species with increasing phylogenetic distance that is expected for several microevolutionary processes, including drift and directional selection, whereas an O-U process models the exponential decrease expected for traits experiencing stabilizing selection (Hansen and Martins 1996). For datasets for which sequences were not available or could not be reliably aligned, but for which a topology was available from other data, a speciation model was applied with phylogeny branch lengths set to one. Phenotypic covariance in this model is expected to decrease linearly as for other Brownian motion models (Hansen and Martins 1996). Where available, the models also incorporated standard error estimates for the enzyme data into the variance components of the error term. The statistical significance of the correlation coefficients was assessed by whether the confidence interval for the slope of the regression line overlapped zero, per the authors' recommendation.

Statistical models

r values were rescaled by $r/2 + 0.5$ to take on values between 0 and 1 and then logit-transformed to make the data approximately normal. The normality of the transformed distributions was assessed with Shapiro–Wilk tests. Linear random effect and mixed models of the rescaled logit-transformed data were fit by REML using Jmp version 10 (SAS Institute).

For datasets for which nucleotide sequences were available for enzyme-coding genes, average synonymous nucleotide divergence K_s was estimated by the Nei-Gojobori method using DnaSP version 5 (Librado and Rozas 2009). Samples were assigned to one of two divergence classes based on whether the average K_s was less than or greater than 0.25 synonymous nucleotide substitutions per synonymous site. The average difference in temperature between a pair of sampled enzymes was also estimated for datasets with information on the optimal environmental temperature of the organisms from which the sampled enzymes were derived.

Stability-activity relationship for the directed evolution of engineered enzymes

To investigate the stability-activity relationship for engineered enzymes created by random mutagenesis, I conducted a literature search between 1995 and 2016 for the search terms "directed evolution" and "stability." To be included in the analysis, a study was required to comprehensively screen at least stability or activity of mutant enzymes and subsequently assess for a trade-off in the other trait.

Results and Discussion

Datasets and phylogenetic comparative methods

Because the sensitivity of enzyme performance to reaction conditions complicates comparative biochemistry across independent investigations, only data obtained from the same study were considered. To be included, data were required for three or more purified orthologous enzymes for some measure of both (1) enzyme stability and (2) enzyme kinetics assayed at a common garden temperature. For the former, this included enzyme denaturation temperature (T_m), enzyme optimal temperature (T_{opt}), time to 50% enzyme inactivation (T_{50}) or other estimate of residual activity, and the free energy of unfolding (ΔG_U). For the latter, this included enzyme catalytic turnover number (k_{cat}), maximal rate of catalysis (V_{max}), enzyme specific activity, and the Michaelis constant (K_m), a measure of an enzyme's substrate affinity ($1/K_m$

is related but not identical to enzyme-binding affinity). A negative relationship between these stability and activity metrics is consistent with a trade-off based on the proposed mechanism that a more stable and rigid enzyme both binds its substrate(s) more tightly (lower K_m) and is a slower catalyst (lower k_{cat} etc.). Datasets were obtained from Web of Science searches from 1968 (the year of Hochachka and Somero's landmark paper on temperature adaptation of enzymes; Hochachka and Somero 1968) through 2016 under the terms (1) "enzyme stability" and "temperature" and (2) "temperature adaptation" and "protein," respectively.

Fifty-two datasets from 34 studies for the stability-activity relationship (Table 1) were identified for analysis. Given the rich respective histories of research on both thermal physiology and enzyme biochemistry, it is perhaps surprising how few studies met the above criteria; frequently, comparative biochemical studies only involved two enzymes, typically isolated from anciently divergent taxa.

Comparative phenotypic data from related organisms are expected to violate the assumptions of standard statistical models in a manner analogous to the degrees of freedom problem associated with pseudo-replication, thereby giving false confidence in significance tests. Therefore, to conduct correlation analyses, these assumptions were relaxed with a phylogenetic generalized least squares (PGLS) approach (Martins and Hansen 1997), which accounts for the statistical dependence of comparative data in the error term of the model by converting phylogeny branch lengths into units of expected phenotypic divergence (Methods). When possible, these branch lengths were estimated from sequence data for each enzyme in the sample; alternatively, for samples for which sequences were not available or could not be reliably aligned, but for which a topology was available from other data, a speciation model was employed in which branch lengths were set equal to one.

Studies of more divergent enzymes are more likely to exhibit a negative relationship between stability and activity

Thirty-nine of the 52 stability-activity PGLS models estimated the predicted negative correlation (mean $r = 0.30$), though estimated values varied greatly and, in most cases, the correlation was weak and not significant (Fig. 1; Table 1). Because many studies had low sample size, it is difficult to distinguish the absence of a relationship from a statistical power issue. For further statistical analysis, estimated r values were rescaled and then logit-transformed to make them approximately normal as assessed by the Shapiro–Wilk test ($P = 0.17$). A linear model with these transformed data as the response variable and with study as random effect indicated that a majority of the variation in estimated correlations could be explained by differences among studies (57.0% of the total REML variance component estimate).

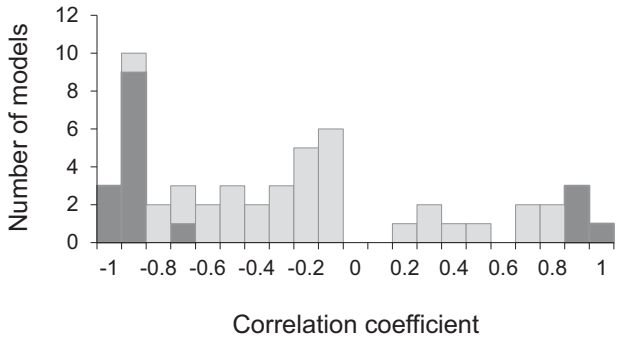

Figure 1. Frequency distribution of correlation coefficients estimated by phylogenetic generalized least squares for 52 models of the stability-activity relationship indicated in Table 1. An estimated negative correlation between stability and activity is consistent with the prediction of a trade-off mechanism. Darker shaded values indicate estimated correlations that are significantly different from zero.

To further explore the factors contributing to this variation among studies, two linear mixed models were developed. The first incorporated sequence divergence as a fixed effect. The average amount of synonymous nucleotide sequence divergence (K_s, a proxy for the passage of time) varied dramatically among studies for which sequence data were available for the sampled enzymes, with a majority of studies focused on deeply divergent enzymes. Studies were assigned to one of two divergence classes based on whether K_s between enzyme-coding genes was less than or greater than 0.25 synonymous nucleotide substitutions per synonymous site ("low" and "high" divergence, respectively; Table 1). By this criterion, 11 estimated correlations from seven studies were classified as "low divergence" and 28 from 18 studies as "high divergence." The second model included the average difference in organism optimal environmental temperature as a fixed effect for studies for which ecological data were available for sampled taxa: one might expect that stability and activity might be more strongly associated for studies investigating enzymes derived from more divergent thermal environments.

The effect of sequence divergence class on the stability–activity relationship was very highly significant ($F_{[1,21.88]} = 13.73$; $P = 0.001$; $N = 38$), with studies of more divergent enzymes more likely to exhibit a negative relationship between stability and activity (Fig. 2). This model fit the data better than a null model with only study as random effect, based on lower AICc and BIC scores (ΔAICc $= 8.36$, ΔBIC $= 7.23$). By contrast, there was no effect of the average difference in optimal environmental temperature ($F_{[1,17.6]} = 1.07$; $P = 0.32$; $N = 37$) on the stability–activity relationship. That is, the relationship did not depend on the amount of ecological divergence among taxa, nor was the above association with sequence divergence a product of more divergent enzymes being derived from organisms from more divergent habitats.

Table 1. Stability-activity relationship models analyzed in this study.

Enzyme	Model	N[1]	r^2	Divergence Class[3]	Temperature Difference[4]	Reference
malate dehydrogenase	Specific activity v. enzyme T_{opt}	8	−0.4	-[5]	–	Wali et al. 1979
alcohol dehydrogenase	$K_{m[ethanol]}$ v. residual activity	7	−0.25	High	–	Alahiotis 1982
lactate dehydrogenase	k_{cat} v. T_{50}	3	−0.50	High	20	Zülli et al. 1991
	$K_{m[pyruvate]}$ v. T_{50}		−0.70			
DNA polymerase	$K_{m[dNTP]}$ v. T_{inact}	4	**−0.94**	–	8	Sellmann et al. 1992
B4-lactate dehydrogenase	$K_{m[pyruvate]}$ v. T_{50}	3	−0.83	High	0	Voorter et al. 1993
	$K_{m[NADH]}$ v. T_{50}		−0.13			
malate dehydrogenase	$K_{m[NADH]}$ v. log residual activity	5	−0.56	–	6	Dahlhoff and Somero 1993
3-methylaspartase	Specific activity v. enzyme T_{opt}	3	−0.11	–	0	Kato and Asano 1995
	$K_{m[3\text{-methylaspartic acid}]}$ v. enzyme T_{opt}		−0.30			
A_4-lactate dehydrogenase	$K_{m[pyruvate]}$ v. T_{50}	3	0.39	Low	15	Fields and Somero 1997
A-lactate dehydrogenase	$K_{m[pyruvate]}$ v. residual activity	3	0.80	Low	4	Holland et al. 1997
	$K_{m[NADH]}$ v. residual activity		0.81			
A_4-lactate dehydrogenase	k_{cat} v. residual activity	6	−0.19	Low	2	Fields and Somero 1998
	$K_{m[pyruvate]}$ v. residual activity	10	0.28			
RuBisCO	Specific activity v. T_{50}	3	**0.90**	–	14	Devos et al. 1998
Phosphoglycerate kinase	k_{cat} v. enzyme T_{opt}	3	0.99	–	27	Thomas and Scopes 1998
	$K_{m[3\text{-phosphoglyceric acid}]}$ v. enzyme T_{opt}		0.65			
malate dehydrogenase	k_{cat} v. T_m	3	−1.0	High	44	Kim et al. 1999
	$K_{m[oxaloacetate]}$ v. T_m		−0.96			
indoleglycerol phosphate synthase	k_{cat} v. T_{50}	3	−0.97	High	32	Merz et al. 1999
	$K_{m[1\text{-(o-carboxyphenylamino-1-deoxyribulose-5-phosph}$ v. T_{50}		−0.94			
phytase	Specific activity v. enzyme T_{opt}	6	−0.25	High	2	Wyss et al. 1999
phosphoglucose isomerase	$K_{m[F6P]}$ v. Arrhenius break temp.	3	**−1.0**	–	–	Dahlhoff and Rank 2000
3-isopropylmalate dehydrogenase	k_{cat} v. T_m	3	**−0.99**	–	37	Svingor et al. 2001
phytase	Specific activity v. T_m	5	−0.37	High	0	Lassen et al. 2001
lactate dehydrogenase	k_{cat} v. T_{50}	6	0.44	High	–	Sharpe et al. 2001
	$K_{m[pyruvate]}$ v. T_{50}		0.30			

(*Continued*)

Table 1. Continued.

Enzyme	Model	N[1]	r^2	Divergence Class[3]	Temperature Difference[4]	Reference
α-amylase	k_{cat} v. enzyme T_{opt}	3	**−0.96**	High	15	D'Amico et al. 2003
α-amylase	$K_{m[starch]}$ v. T_{50}	3	−0.40	High	7	Adewale et al. 2006
	V_{max} v. T_{50}		−0.43			
isocitrate dehydrogenase	$K_{m[isocitrate]}$ v. enzyme T_{opt}	3	−0.24	High	47	Fedøy et al. 2007
	$K_{m[NADP+]}$ v. enzyme T_{opt}		−0.66			
	k_{cat} v. enzyme T_{opt}		−0.88			
lactate dehydrogenase	k_{cat} isotherm v. T_m	3	**−0.90**[6]	High	47	Coquelle et al. 2007
alcohol dehydrogenase	V_{max} v. enzyme T_{opt}	3	**−0.99**	High	30	Barzegar et al. 2009
thermolysin	k_{cat}/K_m v. T_{opt}	3	−0.77	High	17	Xie et al. 2009
malate dehydrogenase	$K_{m[NADH]}$ v. residual activity	6	−0.28	Low	–	Dong and Somero 2009
pyruvate decarboxylase	V_{max} v. enzyme T_{opt}	3		High	3	Gocke et al. 2009
	$K_{m[pyruvate]}$ v. enzyme T_{opt}		−0.54 0.19			
dihydrofolate reductase	k_{cat} v. ΔG_U	7	−0.74	High	–	Murakami et al. 2011
	$K_{m[dihydrofolate]}$ v. ΔG_U		−0.14			
α-amylase	$K_{m[starch]}$ v. enzyme T_{opt}	3	**−1.0**	High	10	Mahdavi et al. 2011
α-amylase	$K_{m[Et\text{-}G7\text{-}pNP]}$ v. Tm	4	0.89	–	28	Cipolla et al. 2013
	k_{cat} v. Tm		**−0.98**			
arylalkylamine N-acetyltransferase	$K_{m[tryptamine]}$ v. residual activity	3	0.63	Low	–	Cazaméa-Catalan et al. 2013
	k_{cat} v. residual activity		−0.23			
feruloyl esterase	$K_{m[methyl\ sinapate]}$ v. enzyme T_{opt}	6	−0.37	High	–	Rashamuse et al. 2014
	k_{cat} v. enzyme T_{opt}		−0.12			
uronate dehydrogenase	k_{cat} v. enzyme T_{opt}	5	0.71	–	–	Pick et al. 2015
	$K_{m[glucoronate]}$ v. enzyme T_{opt}		−0.68			
esterase	k_{cat} v. enzyme T_{opt}	4	−0.92	High	–	Kovacic et al. 2016
cuproxidase	V_{max} v. T_m	3	−0.17	High	–	Roulling et al. 2016

[1]Number of enzymes in sample.

[2]Statistically significant *r* values are in bold.

[3]Low : < 0.25 synonymous nucleotide substitutions per synonymous site; High: > 0.25 synonymous nucleotide substitutions per synonymous site.

[4]Average difference in physiological temperature optimum or environmental temperature.

[5]Data not available.

[6]For this model, the estimated positive correlation between these variables is expected for a trade-off. The sign of the correlation coefficient was therefore changed for comparison with the other models.

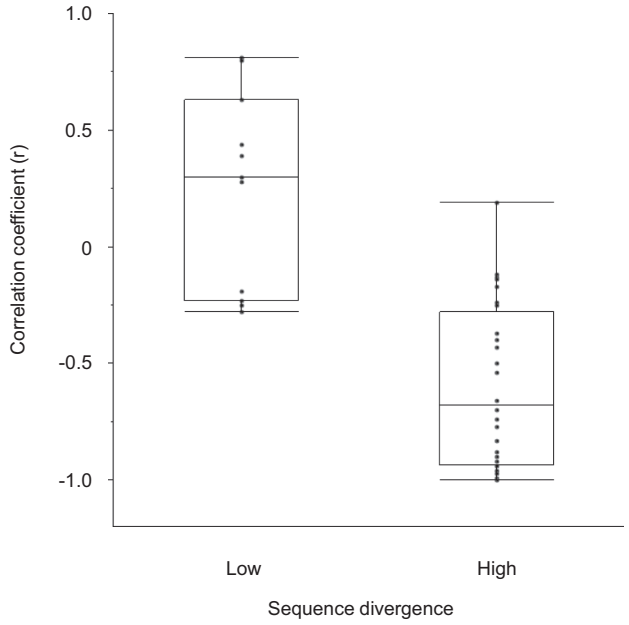

Figure 2. Box plots of correlation coefficients for models of the stability-activity relationship for which the mean synonymous nucleotide divergence K_s of the sampled enzymes was either less than ("Low") or greater than ("High") 0.25 substitutions per synonymous nucleotide site, respectively.

This result potentially has important implications for our understanding of the mechanisms underlying the relationship between stability and activity for natural enzymes. For a trade-off mechanism mediated by the pleiotropic effects of changes in enzyme flexibility on both stability and function, a negative relationship is expected to be intrinsic to the process of enzyme functional divergence itself and therefore independent of the level of sequence divergence. By contrast, the observed effect of sequence divergence suggests that, for some enzymes, a negative relationship between stability and activity may only develop over time as enzymes diverge, rather than by a trade-off mechanism. What might be responsible for this pattern? One possibility is that this may instead be due to the accumulation of conditionally deleterious mutations that are not exposed to selection in the native environment but which are revealed during activity assays at lower temperature.

Do we see any evidence for the emergence of a negative relationship between stability and activity as natural enzymes diverge? Although no single dataset in this analysis can address this question, there are six different lactate dehydrogenase studies spanning a wide range of mean sequence divergence ($K_s = 0.009$–0.375 synonymous nucleotide substitutions/synonymous site) for which data are available for both $K_{m[pyruvate]}$ and enzyme stability (Table 1; Zülli et al. 1991; Voorter et al. 1993; Holland et al. 1997; Fields and Somero 1997; Fields and Somero 1998; Sharpe et al. 2001). Consistent with mutation accumulation as opposed to a direct trade-off mechanism, the estimated correlations are positive for samples with lower mean sequence divergence and become more negative with increasing divergence ($r = -0.76$ for the relationship between r_{study} and K_s; $F_{[1,4]} = 5.52$, $P = 0.078$).

Engineered enzymes frequently do not exhibit a stability-activity trade-off

Another possible source of insight on this issue may be found in the biotechnology literature. Twenty-three directed evolution studies were identified for which mutant enzymes engineered by the random mutagenesis of a wild-type template were (1) screened for an increase in either stability and/or activity compared with the wild type and (2) subsequently assessed for a trade-off in the other trait (Table 2). The majority of these studies involved a single generation of error-prone PCR under conditions for which only 1–2 amino acid changes were expected per mutant. Though designed as a high-throughput strategy for identifying changes in enzyme properties of potential commercial interest, the approach informs our understanding of the evolutionary process itself by providing samples of functionally divergent enzymes that are nearly identical in sequence. Mutants with enhanced performance for the screened trait were generally rare, in accord with the expectation that most amino acid substitutions are deleterious. However, for many studies, among those mutants with an evolved increase in either thermal stability or activity, there was often no cost in performance for the other trait (Table 2). Therefore, at microevolutionary scales of divergence, a direct trade-off between stability and activity frequently does not exist for either laboratory-evolved or natural enzymes.

The frequent absence of a stability-activity trade-off during microevolutionary divergence is not restricted to an in vitro directed evolution approach. Often, enzyme thermostability also significantly increases without an activity cost during the in vivo experimental evolution of laboratory populations of a genetically modified thermophile that has been rendered temperature sensitive by the replacement of a native enzyme with a less stable ortholog (Akanuma et al. 1998; Tamakoshi et al. 2001; Couñago et al. 2006; Nakamura et al. 2008). For example, selection for increased stability of the leucine biosynthetic enzyme 3-isopropylmate dehydrogenase in a leucine-auxotrophic strain of the thermophilic host bacterium *Thermus thermophilus* resulted in a more stable enzyme without a cost in activity, irrespective of whether the less stable progenitor enzyme was derived from the mesophilic bacterium *Bacillus subtilis* (Akanuma et al. 1998) or from yeast (Tamakoshi et al. 2001). Similarly, following the replacement of adenylate kinase from *Geobacillus stearothermophilus* with that of *B. subtilis*, a series of more stable but not necessarily less active enzyme variants evolved during the course of 1500 *G. stearothermophilus* generations under increasing temperature (Couñago et al. 2006). Notably, structural analysis of one

Table 2. Stability-activity relationship for enzymes directed evolved by random mutagenesis.

Enzyme	Method[1]	Screen		No. improved	No. improved without cost (%)	Reference
		No. of mutants	Trait			
subtilisin S41	epPCR	864	Stability	23	12 (52.2)	Miyazaki and Arnold 1999
subtilisin E	epPCR	5,000	Stability, activity	5	5 (100)	Zhao and Arnold 1999
maltogenic α-amylase	DNA-S	1,500	Stability	2	2 (100)	Kim et al. 2003
prolyl endopeptidase	epPCR	10,752	Stability	1	1 (100)	Heinis et al. 2004
esterase	epPCR	2,500	Stability	23	5 (21.7)	Kim et al. 2004
xylanase	GSSM	70,000	Stability	9	4 (44.4)	Palackal et al. 2004
phosphite dehydrogenase	epPCR	3,200	Stability	5	5 (100)	Johannes et al. 2005
pectate lyase	GSSM	13,000	Stability	12	1 (8.3)	Solbak et al. 2005
Type L α-glucan phosphorylase	epPCR	25,000	Stability	3	3 (100)	Yanase et al. 2005
amylosucrase	MutaGen	60,000	Stability	3	3 (100)	Emond et al. 2008
		30,000	Stability	2	2 (100)	
alkaline phosphatase	epPCR	13,000	Stability	3	0 (0)	Koutsioulis et al. 2008
tyrosine phenol-lyase	epPCR	12,000	Activity	4	1 (25.0)	Rha et al. 2009
		12,000	Stability	3	2 (66.7)	
formaldehyde dehydrogenase	epPCR	2,300	Stability	11	2 (18.2)	Imamura and Shigemori 2010
phloroglucinol synthase	epPCR	3,000	Stability	7	7 (100)	Rao et al. 2013
formate dehydrogenase	epPCR	987	Activity	1	1 (100)	Carter et al. 2014
lipase	epPCR	1,500	Stability	3	3 (100)	Madan and Mishra 2014
phytase	epPCR	96	Activity	2	2 (100)	Chen et al. 2015
glucose oxidase	epPCR	2,800	Stability	1	1 (100)	Marin-Navarro et al. 2015
α-glucosidase	epPCR	2,700	Stability	1	0 (0)	Zhou et al. 2015
esterase	epPCR	8,000	Stability	1	1 (100)	Jiang et al. 2016
xylanase	epPCR	5,000	Stability	4	1 (25)	bin Abdul Wahab et al. 2016

[1]Abbreviations: DNA-S – DNA shuffling; epPCR – error-prone PCR; GSSM - gene site saturation mutagenesis

mutant for which there was a decrease in activity at lower temperatures (and which differed from the *B. subtilis* enzyme by only a single amino acid) revealed the source of enhanced stability to be new ionic interactions that reduce enzyme flexibility (Couñago et al. 2008), precisely the thermodynamic mechanism for which a trade-off is expected.

Concluding remarks

Though the existence of a stability-activity trade-off is often asserted for natural enzymes, the evolution of stability, and activity may clearly be uncoupled during enzyme divergence. The present study emphasizes that the general importance of the trade-off remains to be established. This is, in part, because comparative enzymological data that address this issue are limited. Moreover, it is also due to the possibility that, for datasets focused on highly divergent enzymes, the observed negative relationship between stability and activity that is predicted by a trade-off mechanism may have originated by an alternative process. To distinguish a direct stability-activity trade-off from an alternative mechanism, I recommend that future investigations of this issue focus on samples of recently divergent enzymes (for example between recently divergent populations or species). This would avoid the possible indirect effects of long-term divergence in different thermal environments on enzyme stability and function (Fig. 2) and thereby facilitate the inference of the actual mechanism underlying the stability–activity relationship during the process of ecological diversification.

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AUTHOR CONTRIBUTIONS

S. R. Miller designed the study, organized data, performed analyses and wrote the article.

DATA ARCHIVING

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LITERATURE CITED

Adewale, I., E. Agumanu, and F. Otih-Okoronkwo. 2006. Comparative studies on α-amylases from malted maize (*Zea mays*), millet (*Eleusine coracana*) and sorghum (*Sorghum bicolor*). Carbohydr. Polym. 66:71–74.

Akanuma, S., A. Yamagishi, N. Tanaka, and T. Oshima. 1998. Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. Protein. Sci. 7:698–705.

Alahiotis, S. 1982. Adaptation of *Drosophila* enzymes to temperature IV. Natural selection at the alcohol-dehydrogenase locus. Genetica 59:81–87.

Arnold, F. H., P. Wintrode, K. Miyazaki, and A. Gershenson. 2001. How enzymes adapt: lessons from directed evolution. Trends Biochem. Sci. 26:100–106.

Barzegar, A., A. Moosavi-Movahedi, J. Z. Pedersen, and M. Miroliaei. 2009. Comparative thermostability of mesophilic and thermophilic alcohol dehydrogenases: stability-determining roles of proline residues and loop conformations. Enzyme Microb. Technol. 45:73–79.

Becktel, W., and J. Schellman. 1987. Protein stability curves. Biopolymers 26:1859–1877.

bin Abdul Wahab, M. K. H., M. A. bin Jonet, and R. M. Illias. 2016. Thermostability enhancement of xylanase *Aspergillus fumigatus* RT-1. J. Mol. Catal. B: Enzym. 134:154–163.

Carter, J. L. L., M. Bekhouche, A. Noiriel, L. J. Blum, and B. Douméche. 2014. Directed evolution of a formate dehydrogenase for increased tolerance to ionic liquids reveals a new site for increasing the stability. Chembiochem 15:2710–2718.

Cazaméa-Catalan, D., E. Magnanou, R. Helland, L. Besseau, G. Boeuf, J. Falcón, and E. H. Jørgensen. 2013. Unique arylalkylamine *N*-acetyltransferase-2 polymorphism in salmonids and profound variations in thermal stability and catalytic efficiency conferred by two residues. J. Exp. Biol. 216:1938–1948.

Chen, W., L. Ye, F. Guo, Y. Lv, and H. Yu. 2015. Enhanced activity of an alkaline phytase from *Bacillus subtilis* 168 in acidic and neutral environments by directed evolution. Biochem. Eng. J. 98:137–143.

Chown, S. L., A. A. Hoffmann, T. N. Kristensen, M. J. Angilletta, N. C. Stenseth, and C. Pertoldi. 2010. Adapting to climate change: a perspective from evolutionary physiology. Climate Res. 43:3–15.

Cipolla, A., F. Delbrassine, J.-L. Da Lage, and G. Feller. 2013. Temperature adaptations in psychrophilic, mesophilic and thermophilic chloride-dependent alpha-amylases. Biochimie 94:1943–1950.

Coquelle, N., E. Fioravanti, M. Weik, and F. Vellieux. 2007. Activity, stability and structural studies of lactate dehydrogenases adapted to extreme thermal environments. J. Mol. Biol. 374:547–562.

Couñago, R., S. Chen, and Y. Shamoo. 2006. In vivo molecular evolution reveals biophysical origins of organismal fitness. Mol. Cell. 22:441–449.

Couñago, R., C. J. Wilson, M. I. Peña, P. Wittung-Stafshede, and Y. Shamoo. 2008. An adaptive mutation in adenylate kinase that increases organismal fitness is linked to stability-activity trade-offs. Protein Eng. Des. Sel. 21:19–27.

Dahlhoff, E., and N. Rank. 2000. Functional and physiological consequences of genetic variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme genotype in a montane beetle. Proc. Natl. Acad. Sci. USA 97:10056–10061.

Dahlhoff, E., and G. N. Somero. 1993. Kinetic and structural adaptations of cytoplasmic malate dehydrogenases of eastern Pacific abalone (genus *Haliotis*) from different thermal habitats: biochemical correlates of biogeographical patterning. J. Exp. Biol. 185:137–150.

D'Amico, S., J.-C. Marx, C. Gerday, and G. Feller. 2003. Activity-stability relationships in extremophilic enzymes. J. Biol. Chem. 278:7891–7896.

Daniel, R. M., R. V. Dunn, J. L. Finney, and J. C. Smith. 2003. The role of dynamics in enzyme activity. Annu. Rev. Biophys. Biomol. Struct. 32:69–92.

DePristo, M. A., D. M. Weinreich, and D. L. Hartl. 2005. Missense meanderings in sequence space: a biophysical view of protein evolution. Nat. Rev. Genet. 6:678–687.

Devos, N., M. Ingouff, R. Loppes, and R. F. Matagne. 1998. Rubisco adaptation to low temperatures: a comparative study in psychrophilic and mesophilic unicellular algae. J. Phycol. 34:655–660.

Dong, Y., and G. N. Somero. 2009. Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus *Lottia*): differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions. J. Exp. Biol. 212:169–177.

Elias, M., G. Wieczorek, S. Rosenne, and D. S. Tawfik. 2014. The universality of enzymatic rate-temperature dependency. Trends Biochem. Sci. 39: 1–7.

Emond, S., I. André, K. Jaziri, G. Potocki-Véronèse, P. Mondon, K. Bouayadi, H. Kharrat, P. Monsan, and M. Remaud-Simeon. 2008. Combinatorial engineering to enhance thermostability of amylosucrase. Protein Sci. 17:967–976.

Fedøy, A., N. Yang, A. Martinez, H.-K. Leiros, and I. H. Steen. 2007. Structural and functional properties of isocitrate dehydrogenase from the psychrophilic bacterium *Desulfotalea psychrophila* reveal a cold-active enzyme with an unusual high thermal stability. J. Mol. Biol. 372:130–149.

Feller, G. 2010. Protein stability and enzyme activity at extreme biological temperatures. J. Phys. Condens. Matter 22:323101.

Fields, P. A., and G. N. Somero. 1997. Amino acid sequence differences cannot fully explain interspecific variation in thermal sensitivities of gobiid fish A4-lactate dehydrogenases (A4-LDHs). J. Exp. Biol. 200:1839–1850.

Fields, P. A., and G. N. Somero. 1998. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A_4 orthologs of Antarctic notothenioid fishes. Proc. Natl. Acad. Sci. USA 95:11476–11481.

Fields, P. A. 2001. Review: protein function at thermal extremes: balancing stability and flexibility. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129:417–431.

Garcia-Viloca, M., J. Gao, M. Karplus, and D. G. Truhlar. 2004. How enzymes work: analysis by modern rate theory and computer simulations. Science 303:186–195.

Gocke, D., T. Graf, H. Brosi, I. Frindi-Wosch, L. Walter, M. Müller, and M. Pohl. 2009. Comparative characterisation of thiamin diphosphate-dependent decarboxylases. J. Mol. Catal. B Enzym. 61:30–35.

Hammes-Schiffer, S., and S. J. Benkovic. 2006. Relating protein motion to catalysis. Annu. Rev. Biochem. 75:519–541.

Hansen, T. F., and E. P. Martins. 1996. Translating between microevolutionary process and macroevolutionary patterns: the correlation structure of interspecific data. Evolution 50:1404–1417.

Hart, K. M., M. J. Harms, B. H. Schmidt, C. Elya, J. W. Thornton, and S. Marqusee. 2014. Thermodynamic system drift in protein evolution. PLoS Biol. 12:e1001994.

Heinis, C., P. Alessi, and D. Neri. 2004. Engineering a thermostable human prolyl endopeptidase for antibody-directed enzyme prodrug therapy. Biochemistry 43:6293–6303.

Henzler-Wildman, K. A., M. Lei, V. Thai, S. J. Kerns, M. Karplus, and D. Kern. 2007. A hierarchy of timescales in protein dynamics is linked to enzyme catalysis. Nature 450:913–916.

Hernández, G., F. E. Jenney, M. W. Adams, and D. M. LeMaster. 2000. Millisecond time scale conformational flexibility in a hyperthermophile protein at ambient temperature. Proc. Natl. Acad. Sci. USA 97:3166–3170.

Hochachka, P. W., and G. N. Somero. 1968. The adaptation of enzymes to temperature. Comp. Biochem. Physiol. 27:659–668.

Hochachka, P. W., and G. N. Somero. 2002. Biochemical adaptation: mechanism and process in physiological evolution. Oxford Univ. Press, Oxford, U. K.

Holland, L. Z., M. McFall-Ngai, and G. N. Somero. 1997. Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus *Sphyraena*) from different thermal environments: differences in kinetic properties and and thermal stability are due to amino acid substitutions outside the active site. Biochemistry 36:3207–3215.

Imamura, C., and Y. Shigemori. 2010. Enhancement of thermal stabilization of formaldehyde dehydrogenase from *Pseudomonas putida* by directed evolution. Biosci. Biotechnol. Biochem. 74:1462–1465.

Jaenicke, R., and P. Závodszky. 1990. Proteins under extreme physical conditions. FEBS Lett. 268:344–349.

Jaenicke, R. 1991. Protein stability and molecular adaptation to extreme conditions. Eur. J. Biochem. 202:715–728.

Jaenicke, R. 2000. Do ultrastable proteins from hyperthermophiles have high or low conformational rigidity? Proc. Natl. Acad. Sci. USA 97:2962–2964.

Jiang, H., S. Zhang, H. Gao, and N. Hu. 2016. Characterization of a cold-active esterase from *Serratia* sp. and improvement of thermostability by directed evolution. BMC Biotechnol. 16:7.

Johannes, T. W., R. D. Woodyer, and H. Zhao. 2005. Directed evolution of a thermostable phosphite dehydrogenase for NAD(P)H regeneration. Appl. Environ. Microbiol. 71:5728–5734.

Karshikoff, A., L. Nilsson, and R. Ladenstein. 2015. Rigidity versus flexibility: the dilemma of understanding protein thermal stability. FEBS J. 282:3899–3917.

Kato, Y., and Y. Asano. 1995. Purification and properties of crystalline 3-methylaspartase from two facultative anaerobes, *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601. Biosci. Biotechnol. Biochem. 59:93–99.

Kim, J., G. Choi, S. Kim, W. Kim, and J. Lee. 2004. Enhanced thermostability and tolerance of high substrate concentration of an esterase by directed evolution. J. Mol. Catal. B Enzym. 27:169–175.

Kim, S. Y., K. Y. Hwang, S. H. Kim, H. C. Sung, Y. S. Han, and Y. Cho. 1999. Structural basis for cold adaptation sequence, biochemical properties, and crystal structure of malate dehydrogenase from a psychrophile *Aquaspirillium arcticum*. J. Biol. Chem. 274:11761–11767.

Kim, Y. W., J. H. Choi, J. W. Kim, C. Park, H. Cha, S. B. Lee, B. H. Oh, T. W. Moon, and K. H. Park. 2003. Directed evolution of *Thermus* maltogenic amylase toward enhanced thermal resistance. Appl. Environ. Microbiol. 69:4866–4874.

Koutsioulis, D., E. Wang, M. Tzanodaskalaki, D. Nikiforaki, A. Deli, G. Feller, P. Heikinheimo, and V. Bouriotis. 2008. Directed evolution on the cold adapted properties of TAB5 alkaline phosphatase. Protein Eng. Des. Sel. 21:319–327.

Kovacic, F., A. Mandrysch, C. Poojari, B. Strodel, and K.-E. Jaeger. 2016. Structural features determining thermal adaptation of esterases. Protein Eng. Des. Sel. 29:65–76.

Kumar, S., and R. Nussinov. 2001. How do thermophilic proteins deal with heat? Cell Mol. Life. Sci. 58:1216–1233.

Lassen, S. F., J. Breinholt, P. R. Ostergaard, R. Brugger, A. Bischoff, M. Wyss, and C. C. Fuglsang. 2001. Expression, gene cloning, and characterization of five novel phytases from four Basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp., and *Trametes pubescens*. Appl. Environ. Microbiol. 67:4701–4707.

Lazaridis, T., I. Lee, and M. Karplus. 1997. Dynamics and unfolding pathways of a hyperthermophilic and a mesophilic rubredoxin. Protein Sci. 6:2589–2605.

Li, W. T., J. W. Shriver, and J. N. Reeve. 2000. Mutational analysis of differences in thermostability between histones from mesophilic and hyperthermophilic archaea. J. Bacteriol. 182:812–817.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Madan, B., and P. Mishra. 2014. Directed evolution of *Bacillus licheniformis* lipase for improvement of thermostability. Biochem. Eng. J. 91:276–282.

Mahdavi, A., R. Sajedi, S. Asghari, M. Taghdir, and M. Rassa. 2011. An analysis of temperature adaptation in cold active, mesophilic and thermophilic *Bacillus* α-amylases. Int. J. Biol. Macromol. 49:1038–1045.

Marín-Navarro, J., N. Roupain, D. Talens-Perales, and J. Polaina. 2015. Identification and structural analysis of amino acid substitutions that increase the stability and activity of *Aspergillus niger* glucose oxidase. PLoS ONE 10:e0144289.

Martins, E. P., and T. F. Hansen. 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. Am. Nat. 149:646–667.

Merkley, E. D., W. W. Parson, and V. Daggett. 2010. Temperature dependence of the flexibility of thermophilic and mesophilic flavoenzymes of the nitroreductase fold. Protein Eng. Des. Sel. 23:327–336.

Merz, A., T. Knöchel, J. Jansonius, and K. Kirschner. 1999. The hyperthermostable indoleglycerol phosphate synthase from *Thermotoga maritima* is destabilized by mutational disruption of two solvent-exposed salt bridges. J. Mol. Biol. 288:753–763.

Miyazaki, K., and F. H. Arnold. 1999. Exploring nonnatural evolutionary pathways by saturation mutagenesis: rapid improvement of protein function. J. Mol. Evol. 49:716–720.

Murakami, C., E. Ohmae, S. Tate, K. Gekko, K. Nakasone, and C. Kato. 2011. Comparative study on dihydrofolate reductases from *Shewanella* species living in deep-sea and ambient atmospheric-pressure environments. Extremophiles 15:165–175.

Nakamura, A., Y. Takakura, N. Sugimoto, N. Takaya, K. Shiraki, and T. Hoshino. 2008. Enzymatic analysis of a thermostabilized mutant of an *Escherichia coli* hygromycin B phosphotransferase. Biosci. Biotechnol. Biochem. 72:2467–2471.

Palackal, N., Y. Brennan, W. Callen, P. Dupree, G. Frey, F. Goubet, G. P. Hazlewood, S. Healey, Y. E. Kang, K. A. Kretz, et al. 2004. An evolutionary route to xylanase process fitness. Protein Sci. 13:494–503.

Pick, A., J. Schmid, and V. Sieber. 2015. Characterization of uronate dehydrogenases catalysing the initial step in an oxidative pathway. Microb. Biotechnol. 8:633–643.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

Rao, G., J.-K. Lee, and H. Zhao. 2013. Directed evolution of phloroglucinol synthase PhlD with increased stability for phloroglucinol production. Appl. Microbiol. Biotechnol. 97:5861–5867.

Rashamuse, K., T. Ronneburg, W. Sanyika, K. Mathiba, E. Mmutlane, and D. Brady. 2014. Metagenomic mining of feruloyl esterases from termite enteric flora. Appl. Microbiol. Biotechnol. 98:727–737.

Razvi, A., and J. M. Scholtz. 2006. A thermodynamic comparison of HPr proteins from extremophilic organisms. Biochemistry 45:4084–4092.

Rha, E., S. Kim, S. L. Choi, S. P. Hong, M. H. Sung, J. J. Song, and S. G. Lee. 2009. Simultaneous improvement of catalytic activity and thermal stability of tyrosine phenol-lyase by directed evolution. FEBS J. 276:6187–6194.

Risso, V. A., J. A. Gavira, D. F. Mejia-Carmona, E. A. Gaucher, and J. M. Sanchez-Ruiz. 2013. Hyperstability and substrate promiscuity in laboratory resurrections of Precambrian β-lactamases. J. Am. Chem. Soc. 135:2899–2902.

Romero-Romero, M. L., V. A. Risso, S. Martinez-Rodriguez, B. Ibarra-Molero, and J. M. Sanchez-Ruiz. 2016. Engineering ancestral protein hyperstability. Biochem J. 473:3611–3620.

Roulling, F., A. Godin, A. Cipolla, T. Collins, K. Miyazaki, and G. Feller. 2016. Activity-stability relationships revisited in blue oxidases catalyzing electron transfer at extreme temperatures. Extremophiles 20:621–629.

Sawle, L., and K. Ghosh. 2011. How do thermophilic proteins and proteomes withstand high temperature? Biophys. J. 101:217–227.

Sellmann, E., K. Schröder, I. Knoblich, and P. Westermann. 1992. Purification and characterization of DNA polymerases from *Bacillus* species. J. Bacteriol. 174:4350–4355.

Sharpe, M., C. Love, and C. Marshall. 2001. Lactate dehydrogenase from the Antarctic eelpout, *Lycodichthys dearborni*. Polar Biol. 24:258–269.

Solbak, A. I., T. H. Richardson, R. T. McCann, K. A. Kline, F. Bartnek, G. Tomlinson, X. Tan, L. Parra-Gessert, G. J. Frey, M. Podar, et al. 2005. Discovery of pectin-degrading enzymes and directed evolution of a novel pectate lyase for processing cotton fabric. J. Biol. Chem. 280:9431–9438.

Somero, G. N. 1995. Proteins and temperature. Annu. Rev. Physiol. 57:43–68.

Somero, G. N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." J. Exp. Biol. 213:912–920.

Sterner, R., and W. Liebl. 2001. Thermophilic adaptation of proteins. Crit. Rev. Biochem. Mol. Biol. 36:39–106.

Stillman, J. H., and G. N. Somero 2001. A comparative analysis of the evolutionary patterning and mechanistic bases of lactate dehydrogenase thermal stability in porcelain crabs, genus *Petrolisthes*. J. Exp. Biol. 204:767–776.

Svingor, A., J. Kardos, I. Hajdú, A. Németh, and P. Závodszky. 2001. A better enzyme to cope with cold. Comparative flexibility studies on psychrotrophic, mesophilic, and thermophilic IPMDHs. J. Biol. Chem. 276:28121–28125.

Swofford, D. L. 2002. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, MA.

Tamakoshi, M., Y. Nakano, S. Kakizawa, A. Yamagishi, and T. Oshima. 2001. Selection of stabilized 3-isopropylmalate dehydrogenase of *Saccharomyces cerevisiae* using the host-vector system of an extreme thermophile, *Thermus thermophilus*. Extremophiles 5:17–22.

Thomas, M., and K. Scopes. 1998. The effects of temperature on the kinetics and stability of mesophilic and thermophilic 3-phosphoglycerate kinases. Biochem. J. 330:1087–1095.

Thompson, J., D. Higgins, and T. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Trudeau, D. L., M. Kaltenbach, and D. S. Tawfik. 2016. On the potential origins of the high stability of reconstructed ancestral proteins. Mol. Biol. Evol. 33:2633–2641.

Voorter, C., L. Wintjes, P. Heinstra, H. Bloemendal, and W. De Jong. 1993. Comparison of stability properties of lactate dehydrogenase B4/ε-crystallin from different species. Eur. J. Biochem. 211:643–648.

Wali, A., A. Mattoo, and V. Modi. 1979. Comparative temperature-stability properties of malate dehydrogenases from some thermophilic fungi. Int. J. Pept. Protein Res. 14:99–106.

Wheeler, L. C., S. A. Lim, S. Marqusee, and M. J. Harms. 2016. The thermostability and specificity of ancient proteins. Curr. Opin. Struct. Biol. 38:37–43.

Wintrode, P. L., and F. H. Arnold. 2001. Temperature adaptation of enzymes: lessons from laboratory evolution. Adv. Protein Chem. 55:161–225.

Wyss, M., R. Brugger, A. Kronenberger, R. Rémy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl. Environ. Microbiol. 65:367–373.

Xie, B. B., F. Bian, X. L. Chen, et al 2009. Cold adaptation of zinc metalloproteases in the thermolysin family from deep sea and Arctic Sea

ice bacteria revealed by catalytic and structural properties and molecular dynamics: new insights into relationship between conformational flexibility and hydrogen bonding. J. Biol. Chem. 284:9257–9269.

Yanase, M., H. Takata, K. Fujii, T. Takaha, and T. Kuriki. 2005. Cumulative effect of amino acid replacements results in enhanced thermostability of potato type L α-glucan phosphorylase. Appl. Environ. Microbiol. 71:5433–5439.

Závodszky, P., J. Kardos, Á. Svingor, and G. A. Petsko. 1998. Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc. Natl. Acad. Sci. USA 95:7406–7411.

Zhao, H., and F. H. Arnold. 1999. Directed evolution converts subtilisin E into a functional equivalent of thermitase. Protein Eng. 12:47–53.

Zhou, C., Y. Xue, and Y. Ma. 2015. Evaluation and directed evolution for thermostability improvement of a GH 13 thermostable α-glucosidase from *Thermus thermophilus* TC11. BMC Biotechnol. 15:97.

Zülli, F., R. Schneiter, R. Urfer, and H. Zuber. 1991. Structure and function of L-lactate dehydrogenases from thermophilic and mesophilic bacteria, XI. Engineering thermostability and activity of lactate dehydrogenases from Bacilli. Biol. Chem. Hoppe. Seyler. 372:363–372.

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