Journal of Bioscience and Bioengineering
VOL. 128 No. 4, 405–409, 2019

ELSEVIER

www.elsevier.com/locate/jbiosc

# Activity-stability trade-off in random mutant proteins

Ryo Kurahashi, Shun-ichi Tanaka, and Kazufumi Takano*

*Department of Biomolecular Chemistry, Kyoto Prefectural University, Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan*

Received 19 February 2019; accepted 27 March 2019
Available online 12 April 2019

**In our previous study, we investigated the relationship between protein evolution and stability through the random mutational drift of an esterase from hyperthermophilic archaeon *Sulfolobus tokodaii*. The results revealed that evolvability, which is the appearance frequency of variants with higher activity than the parent protein, correlates with parental stability. This suggests that protein evolution that does not take stability into account does not make sense. Here, we used those data to further evaluate the relationship between activity and stability in random mutations, revealing that the maximum increase in activity due to mutation conflicts with parental stability. That is, many activated variants are produced when parental stability is high, whereas lower stability offers a few excellent variants with much higher activity. Moreover, we used the random mutant library to compute a novel criterion, robustizability (stabilizability), which is the appearance frequency of variants with a higher stability than the parent protein. Robustizability correlates positively with parental activity and negatively with parental stability. The results indicated that the principle of activity-stability trade-off dominates, in even random mutations. We propose its application in protein engineering via directed evolution by stability selection.**



[**Key words:** Directed evolution; Esterase; Fitness landscape; Hyperthermophilic archaeon; *Sulfolobus tokodaii*]

Proteins that exist now possess sophisticated functions derived from the surrounding environment through an evolutionary process. They also hold the potential to play an important role in a variety of industrial fields (1–8). However, the functions of existing proteins are not necessarily the best they can be, but are the best for the environments in which their host organisms survive. This can be explained using the concept of a fitness landscape (9,10). Given that fitness is an enzymatic activity, the landscape is probably uneven, with mountains and valleys, and a wild-type protein (WT) would be located somewhere on a mountain in the landscape because it is optimized to its environment (11–13). If this protein is to obtain maximum activity by mutation, it must find a path toward the highest place. In current evolutionary engineering, it is possible for the protein to head to the top of the mountain where it is located (14). However, because of the necessity to go down the mountain once and the huge sequence space, it is difficult for it to reach another neighboring mountain. It would thus seem likely that protein evolution employs some means of drifting in sequence space.

Protein evolution is driven by genetic variations (15–18). Even if mutations are beneficial for evolution, they do not necessarily retain the protein structure to perform a function. Therefore, to accept a variety of mutations, protein stability is thought to be important for protein evolution (19–29). Recently, we investigated the relationship between protein evolution and stability through the random mutational drift of esterases from the hyperthermophilic archaeon *Sulfolobus tokodaii* (Sto-Est) and the hyperthermophilic bacterium *Alicyclobacillus acidocaldarius* (Aac-Est) (30–32). In the experiment of Sto-Est, the evolvability, which is the appearance frequency of variants with higher activity than the parent protein, depends on the parental stability, indicating that protein evolution is governed by protein stability (31). The experimental results for Aac-Est show that the role of protein stability varies with the surrounding environment. High stability is needed for evolution at the optimal temperature while destabilization promotes cold adaptation. This means that protein stability is one factor that decides the direction of protein evolution. Consequently, it is likely that stability is a key to exploring sequence space in the evolutionary process (32).

In this paper, we examine the relationship between protein activity and stability in random mutations based on the data from our previous work (31) to gain a greater understanding of the role of stability in evolution. Here, we defined the ratio of variants that will give a stability greater than the parental protein as robustizability (stabilizability). From the data analysis, the activity-stability trade-off, which is an inherent protein factor, is extracted, revealing a novel relationship between protein evolution and stability and giving useful knowledge for searching sequence space in protein engineering.

## MATERIALS AND METHODS

In the previous report (31), random mutations were introduced to the Sto-Est gene by error-prone PCR to generate an error frequency of one to four substitutions per gene, and then for overproduction, the plasmids of Sto-Est and their derivatives were transformed into *Escherichia coli* BL21-CodonPlus(DE3). A partially purified enzyme was used to perform a simple activity assay, which was then normalized by analyzing the expression level of each target enzyme using SDS-PAGE with ImageJ. Activity and stability analyses of Sto-Est was performed using the

* Corresponding author. Tel./fax: +81 75 703 5654.
*E-mail address:* takano@kpu.ac.jp (K. Takano).

1389-1723/$ – see front matter 
https://doi.org/10.1016/j.jbiosc.2019.03.017

substrate *p*-nitrophenyl butyrate. The assay was carried out iteratively (n = 3). The equations used in this paper are given below. To analyze the stability of Sto-Est against heat treatment, we measured the residual activity at 75°C after incubation at 90°C for 10 min. Relative stability of a variant to WT is expressed as Eq. 1.

$$\text{Relative stability} = \frac{(\text{Activity at } 90^\circ\text{C} \rightarrow 75^\circ\text{C} \,/\text{Activity at } 75^\circ\text{C}) \text{ of variant}}{(\text{Activity at } 90^\circ\text{C} \rightarrow 75^\circ\text{C}/\text{Activity at } 75^\circ\text{C}) \text{ of WT}} \quad (1)$$

Relative activity of a variant to WT Sto-Est is expressed as Eq. 2.

$$\text{Relative activity} = \frac{\text{Activity at } 75^\circ\text{C of variant}}{\text{Activity at } 75^\circ\text{C of WT}} \quad (2)$$

Evolvability and robustizability are calculated by Eqs. 3 and 4, respectively.

$$\text{Evolvability (\%)} = \frac{\text{Number of variants with higher activity than a parent protein}}{\text{Total number of variants}} \times 100 \quad (3)$$

$$\text{Robustizability (\%)} = \frac{\text{Number of variants with higher stability than a parent protein}}{\text{Total number of variants}} \times 100 \quad (4)$$

## RESULTS AND DISCUSSION

**Activity-stability distribution for the variants after random mutations** To examine the relationship between protein activity and stability in the evolutionary process, we performed the experiments for randomly mutated drift with several parental proteins. The parental proteins used in the previous paper (31) were SE-63, WT, SE-63-1, SE-1, SE-6-1, SE-6 and SE-69. Here they are P-1, P-2, P-3, P-4, P-5, P-6 and P-7, respectively, in order of stability. The stability used in this paper is the relative stability with respect to WT, as expressed by Eq. 1. The activity is also the relative activity (Eq. 2). From the results, we plotted the relative activity and stability of the parents and their variant proteins, as shown in Fig. 1A. Generally, the variants are distributed around each parent, except for those from P-7. All P-7 variants further lose both activity and stability compared with P-7, which has already decreased significantly in both activity and stability and does not revive by mutation. This indicates that variants that have serious problems with activity and stability can no longer drift in sequence space, indicating that large deleterious mutations under a certain threshold are eliminated in molecular evolution. Thus P-7 does not fit into the subject of this study, and we therefore exclude it from the following data analysis and discussion.

To make it easy to understand how the features of the parental protein affect the distribution of the variants in each library, the apparent probability ellipses are described in Fig. 1B. Random variants generated from P-6 are distributed slightly toward stabilization and inactivation, but most variants are spread around the parent. On both generations via P-5 and P-4, the change in stability is small while the activity fluctuates significantly (vertical ellipses). This shows that high activity or slight loss of stability tends to produce drastically activity-improved variants. Conversely, parents P-3 and P-1, which have a slight loss of activity parents, show a small change in activity but a large change in stability (horizontal ellipses). Especially, when the parental stability is higher (P-1), the stability of the variants is more widely distributed, indicating that parental stability is an essential factor for accepting various deleterious, neutral and beneficial mutations in protein stability. However, note that no dramatic stabilization was observed from P-1. The results shown in Fig. 1B indicate a trend in the activity-stability distribution that depends on the parental activity and stability. Here, these tendencies may be more prominent depending on the mutation rates.

Next, we focused on variants with maximum activity or maximum stability to understand how proteins improve activity and stability during genetic drift (Fig. 1C). Most variants with improved activity destabilize (upper-left arrows), but P-6 showed only slightly enhanced activity and stability after random mutation (upper-right arrow). This means that a variant must lose stability to improve its activity. Furthermore, the maximum increase in activity due to mutation inversely correlates with parental stability. In other words, the lower the stability, the more dramatically activated mutants are obtained. Note, conversely, that stabilization needs a decrease in activity (lower-right arrow). These results show that the random mutation library is also governed by a trade-off between activity and stability.

**Robustizability in random mutations** There is abundant evidence to support the belief that stability is important for evolution, and our previous work also indicated that evolvability (Eq. 3) correlates positively with parental stability in the original environment (31,32). To examine the role of stability in the evolutionary pathway in more detail, we computed a novel

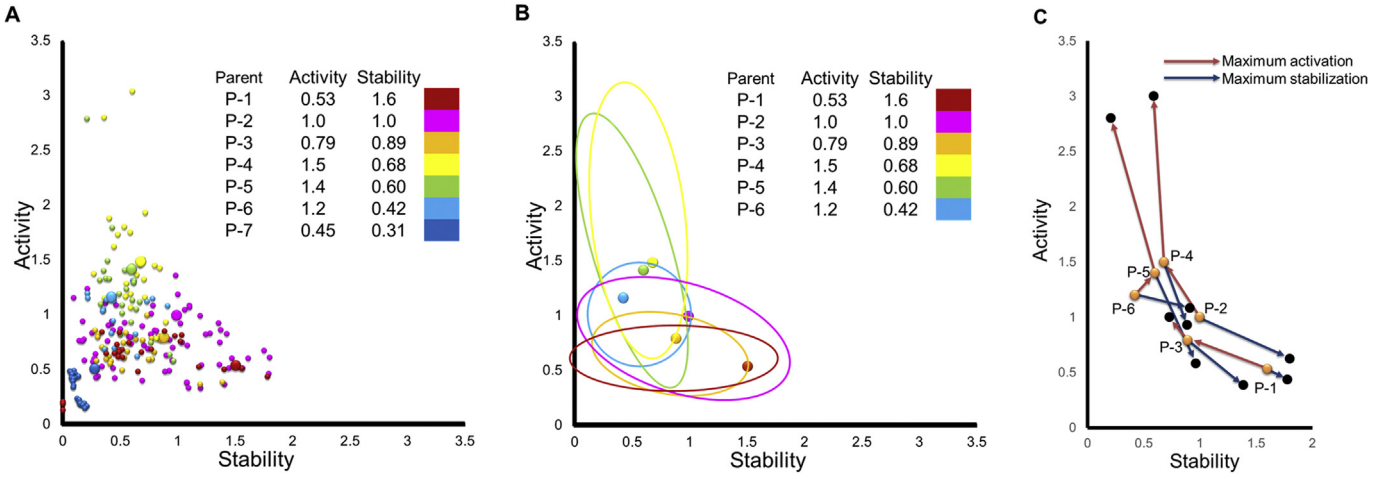


FIG. 1. Variant distribution from each parental protein after random mutations. (A) Parental proteins (P-1, P-2, P-3, P-4, P-5, P-6 and P-7) and their derivatives are colored in red, pink, orange, yellow, yellow green, marine and blue, respectively. The parental proteins are represented in twice larger circles than variants after random mutations. (B) The relationship between each parental protein and its distributed area. This was not obtained by statistical processing. (C) Parents and the maximum activation/stabilization variants color in orange and black, respectively. Evolutionary trajectories toward maximum activation (red arrows) and stabilization (blue arrows) are described.

**TABLE 1.** Characteristics of each parental protein and library.

| Parental protein | Relative activity | Relative stability | Evolvability (%) | Robustizability (%) |
|---|---|---|---|---|
| P-1 | 0.53 | 1.6 | 65 | 7.7 |
| P-2 | 1.0 | 1.0 | 19 | 36 |
| P-3 | 0.79 | 0.89 | 22 | 7.4 |
| P-4 | 1.5 | 0.68 | 41 | 36 |
| P-5 | 1.4 | 0.60 | 17 | 29 |
| P-6 | 1.2 | 0.42 | 17 | 56 |

criterion, robustizability, in each random mutant library (Table 1). Robustizability is the ratio of variants that improve the stability to more than that of the parental protein (Eq. 4), and it can be also referred to as stabilizability. Fig. 2A shows a graph of robustizability against the activity of the parents. Robustizability shows a positive correlation with parental activity. Variants with higher activity form more flexible structures to integrate the substrate, and also have the space to improve stability. Indeed, psychrophilic proteins, which usually have a flexible structure for activity at low temperatures, can easily stabilize (33). Foit et al. (34) found that most variants with improved stability included mutations near the active site on toxin-binding immunity protein 7. Furthermore, we found a negative correlation between robustizability and parental stability (Fig. 2B). This indicates that it is difficult to obtain proteins from a stable protein that are more stable. These results indicate that the activity-stability trade-off dominates even in random mutations.

It is traditionally believed that protein structures reflect two opposite tendencies: (i) the overall structures are organized to be stable; but (ii) their active sites often have flexibility, leading to local instability of active sites. Some experimental studies in early protein engineering indicated that active-site residues are not optimized for conformational stability. Yutani et al. (35) demonstrated that every mutation at Glu49 of the $\alpha$ subunit of tryptophan synthase from *E. coli* reduced the activity significantly, but stabilized the protein. Similar results were found in T4 lysozyme, barnase, citrate synthase, AmpC β-lactamase and ribonuclease HI (36–40). This relationship is termed activity-stability trade-off. Because of the position of this trade-off in protein molecule evolution, destabilization due to high functionality is considered suitable for cold adaptation. As life appears at high temperatures, the trade-off is advantageous in moving to lower temperature environments. The activity-stability trade-off is therefore an essential feature of proteins that they have always possessed, and the characteristics are also extracted in random mutations.

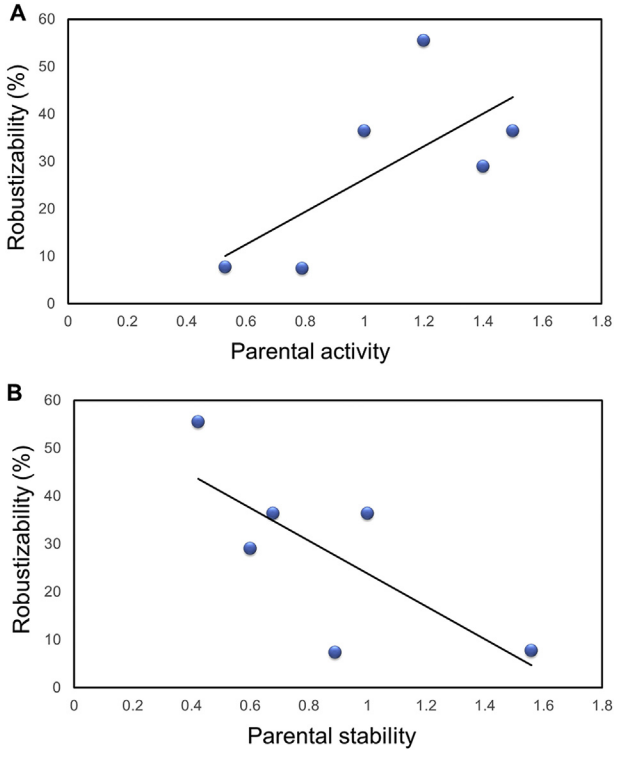


FIG. 2. Relationship between robustizability and parental parameters. Robustizability positively correlated with parental activity (A) and negatively with parental stability (B). Black lines the represent regression lines ($R^2$ = 0.47 and 0.54, respectively).

**Approach for protein engineering** Recently, in the field of protein engineering, the trade-off between protein stability and function has presented a difficult challenge (41,42). As a solution to this issue, chaperone mitigates the effect of destabilization (43–48). A lot more work, including directed evolution experiments equipped with a high-throughput system and a rational design employing computer simulation is needed to offset the trade-off between stability and evolution (49–57).

Generally, experiments of directed evolution for characteristic of an interest are performed selectively using proteins with improved activity, resulting in reaching the peak for increase in activity. That is, in the fitness landscape of activity, it reaches the top of the mountain it is staying (Fig. 3, route A). The problem is that it will cease at the summit and cannot go to other mountains (58–60). Furthermore, since the activity-stability trade-off generally works, the stability of the activating mutants declines (61). To solve the problem, in a previous study we suggested the introduction of protein stability as a selection pressure (31,32), because protein stability is an essential factor in exploring the sequence space to improve activity. However, as described above, even if parental stability is high, this does not mean that the activity of variant proteins will drastically improve. Here, we propose a novel strategy regarding protein stability. First, we perform a stability-indexing experiment on directed evolution to explore sequence space extensively and to help revive activity in the next experiment. At this stage, we select stability-keeping or variants with a slight loss of stability by multiple rounds of random mutations, and generate a large pool of these variants. This pool will contain many variants with different amino acid sequences. These variants will probably have reduced activity due to an activity-stability trade-off, and will be scattered at the foot of various mountains in the fitness landscape (Fig. 3, routes B). Next, as parental proteins, these variants are subjected to the experiment on activity selection. With iterative

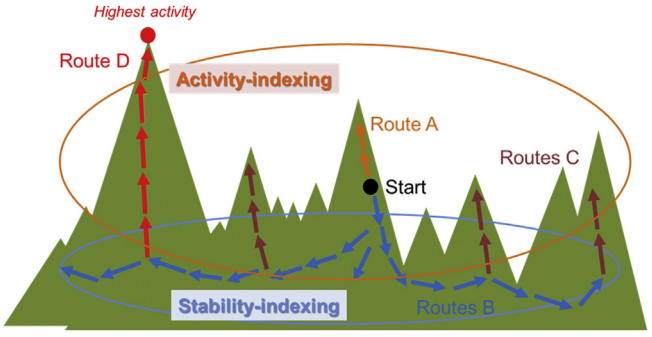


FIG. 3. A novel strategy for getting higher activity mutants. Route A (orange) is the conventional strategy. Routes B (blue) are searching for the foot of the highest mountain in sequence space in the stability-index experiments. Routes C (dark-red) are climbing each mountain by activity-index experiments. Route D (red) is climbing the highest mountain.

experiments, each parent climbs its mountain (Fig. 3, routes C). If a variant is located the foot of the highest mountain, generations of its variants are able to continue to climb to the top, and ultimately, the most active variant will be obtained (Fig. 3, route D). At the second stage, variants are selected according to the activity index as in the conventional method, but instead of selecting the variant with the highest activity from one parent, a highly active variant group is selected from many parents. As can be seen in Fig. 1C, a variant group with slightly increased activity and reduced stability may result in extensive highly-active variants. The point of this two-step selection method is to construct a diverse library of variants in the first stability screen (43). Even at the first stage, the experiments need to be repeated using a mixture of various adaptive variants. It is noted that the similar guideline has been reported; chaperones which buffer the destabilizing effect of mutations allow a protein to explore large sequence space (25,43,44). In this work, we could show the simple concept and strategy by stability-activity index using fitness landscape. We anticipate that this method will be verified experimentally in the future.

In conclusion, this work has demonstrated how parental proteins with various characteristics deploy after random mutations. The results show the trade-off between activity and stability from most aspects. Robustizability, the novel criterion we introduced here, correlates positively with parental activity and negatively with parental stability. These findings have enabled us to suggest a novel approach to improving activity efficiently that can be applied in protein engineering, contributing to industrial fields.

## ACKNOWLEDGMENTS

This work was supported in part by grants from the Japan Society for the Promotion of Science (KAKENHI No. 25440194, 17K07368), and by a Young Researcher Development Support Project from the Kyoto Prefectural Public University Corporation (2018).

## References

1. **Bornscheuer, U. T.:** Microbial carboxyl esterases: classification, properties and application in biocatalysis, FEMS Microbiol. Rev., **26**, 73–81 (2002).
2. **Cavicchioli, R., Siddiqui, K. S., Andrews, D., and Sowers, K.,R.:** Low-temperature extremophiles and their applications, Curr. Opin. Biotechnol., **13**, 253–261 (2002).
3. **Hasan, F., Shah, A. A., and Hameed, A.:** Industrial applications of microbial lipases, Enzyme Microb. Technol., **39**, 235–251 (2006).
4. **Saeki, K., Ozaki, K., Kobayashi, T., and Ito, S.:** Detergent alkaline proteases: enzymatic properties, genes, and crystal structures, J. Biosci. Bioeng., **103**, 501–508 (2007).
5. **Choi, J. M., Han, S. S., and Kim, H. S.:** Industrial applications of enzyme biocatalysis: current status and future aspects, Biotechnol. Adv., **33**, 1443–1454 (2015).
6. **Rahman, M. A., Culsum, U., Tang, W., Zhang, S. W., Wu, G., and Liu, Z.:** Characterization of a novel cold active and salt tolerant esterase from *Zunongwangia profunda*, Enzyme Microb. Technol., **85**, 1–11 (2016).
7. **Madhavan, A., Sindhu, R., Binod, P., Sukumaran, R. K., and Pandey, A.:** Strategies for design of improved biocatalysts for industrial applications, Bioresour. Technol., **245**, 1304–1313 (2017).
8. **Morohoshi, T., Sato, N., Iizumi, T., Tanaka, A., and Ikeda, T.:** Identification and characterization of a novel *N*-acyl-homoserine lactonase gene in *Sphingomonas ursincola* isolated from industrial cooling water systems, J. Biosci. Bioeng., **123**, 569–575 (2017).
9. **Carneiro, M. and Hartl, D. L.:** Adaptive landscapes and protein evolution, Proc. Natl. Acad. Sci. USA, **107**, 1747–1751 (2010).
10. **De Visser, J. A. G. and Krug, J.:** Empirical fitness landscapes and the predictability of evolution, Nat. Rev. Genet., **15**, 480 (2014).
11. **Bershtein, S., Goldin, K., and Tawfik, D. S.:** Intense neutral drifts yield robust and evolvable consensus proteins, J. Mol. Biol., **379**, 1029–1044 (2008).
12. **Romero, P. A. and Arnold, F. H.:** Exploring protein fitness landscapes by directed evolution, Nat. Rev. Mol. Cell Biol., **10**, 866 (2009).
13. **Kaltenbach, M. and Tokuriki, N.:** Dynamics and constraints of enzyme evolution, J. Exp. Zool. B Mol. Dev. Evol., **322**, 468–487 (2014).
14. **Çakar, Z. P., Turanlı-Yıldız, B., Alkım, C., and Yılmaz, Ü.:** Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties, FEMS Yeast Res., **12**, 171–182 (2012).
15. **Kobayashi, R., Hirano, N., Kanaya, S., Saito, I., and Haruki, M.:** Enhancement of the enzymatic activity of *Escherichia coli* acetyl esterase by random mutagenesis, J. Mol. Catal. B Enzym., **67**, 155–161 (2010).
16. **Zhang, S., Wu, G., Feng, S., and Liu, Z.:** Improved thermostability of esterase from *Aspergillus fumigatus* by site-directed mutagenesis, Enzyme Microb. Technol., **64**, 11–16 (2014).
17. **Hang, Y., Ran, S., Wang, X., Jiao, J., Wang, S., and Liu, Z.:** Mutational analysis and stability characterization of a novel esterase of lipolytic enzyme family VI from *Shewanella* sp., Int. J. Biol. Macromol., **93**, 655–664 (2016).
18. **Jiang, H., Zhang, S., Gao, H., and Hu, N.:** Characterization of a cold-active esterase from *Serratia* sp. and improvement of thermostability by directed evolution, BMC Biotechnol., **16**, 7 (2016).
19. **Godoy-Ruiz, R., Perez-Jimenez, R., Ibarra-Molero, B., and Sanchez-Ruiz, J. M.:** Relation between protein stability, evolution and structure, as probed by carboxylic acid mutations, J. Mol. Biol., **336**, 313–318 (2004).
20. **Bloom, J. D., Labthavikul, S. T., Otey, C. R., and Arnold, F. H.:** Protein stability promotes evolvability, Proc. Natl. Acad. Sci. USA, **103**, 5869–5874 (2006).
21. **Bloom, J. D., Raval, A., and Wilke, C. O.:** Thermodynamics of neutral protein evolution, Genetics, **175**, 255–266 (2007).
22. **Camps, M., Herman, A., Loh, E. R. N., and Loeb, L. A.:** Genetic constraints on protein evolution, Crit. Rev. Biochem. Mol. Biol., **42**, 313–326 (2007).
23. **Zeldovich, K. B., Chen, P., and Shakhnovich, E. I.:** Protein stability imposes limits on organism complexity and speed of molecular evolution, Proc. Natl. Acad. Sci. USA, **104**, 16152–16157 (2007).
24. **Elena, S. F. and Sanjuán, R.:** The effect of genetic robustness on evolvability in digital organisms, BMC Evol. Biol., **8**, 284 (2008).
25. **Tokuriki, N. and Tawfik, D. S.:** Stability effects of mutations and protein evolvability, Curr. Opin. Struct. Biol., **19**, 596–604 (2009).
26. **Caetano-Anollés, G. and Mittenthal, J.:** Exploring the interplay of stability and function in protein evolution: new methods further elucidate why protein stability is necessarily so tenuous and stability-increasing mutations compromise biological function, Bioessays, **32**, 655–658 (2010).
27. **Philip, A. F., Kumauchi, M., and Hoff, W. D.:** Robustness and evolvability in the functional anatomy of a PER-ARNT-SIM (PAS) domain, Proc. Natl. Acad. Sci. USA, **107**, 17986–17991 (2010).
28. **Ashenberg, O., Gong, L. I., and Bloom, J. D.:** Mutational effects on stability are largely conserved during protein evolution, Proc. Natl. Acad. Sci. USA, **110**, 21071–21076 (2013).
29. **Wallis, C. P., Richman, T. R., Filipovska, A., and Rackham, O.:** Tighter ligand binding can compensate for impaired stability of an RNA-binding protein, ACS Chem. Biol., **13**, 1499–1505 (2018).
30. **Takano, K., Aoi, A., Koga, Y., and Kanaya, S.:** Evolvability of thermophilic proteins from archaea and bacteria, Biochemistry, **52**, 4774–4780 (2013).
31. **Kurahashi, R., Sano, S., and Takano, K.:** Protein evolution is potentially governed by protein stability: directed evolution of an esterase from the hyperthermophilic archaeon *Sulfolobus tokodaii*, J. Mol. Evol., **86**, 283–292 (2018).
32. **Ota, N., Kurahashi, R., Sano, S., and Takano, K.:** The direction of protein evolution is destined by the stability, Biochimie, **150**, 100–109 (2018).
33. **Wang, X., Minasov, G., and Shoichet, B. K.:** Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs, J. Mol. Biol., **320**, 85–95 (2002).
34. **Foit, L., Morgan, G. J., Kern, M. J., Steimer, L. R., von Hacht, A. A., Titchmarsh, J., Warriner, S. L., Radford, S. E., and Bardwell, J. C. A.:** Optimizing protein stability in vivo, Mol. Cell, **36**, 861–871 (2009).
35. **Yutani, K., Ogasahara, K., Tsujita, T., and Sugino, Y.:** Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit, Proc. Natl. Acad. Sci. USA, **84**, 4441–4444 (1987).
36. **Zhi, W., Srere, P. A., and Evans, C. T.:** Conformational stability of pig citrate synthase and some active-site mutants, Biochemistry, **30**, 9281–9286 (1991).
37. **Meiering, E. M., Serrano, L., and Fersht, A. R.:** Effect of active site residues in barnase on activity and stability, J. Mol. Biol., **225**, 585–589 (1992).
38. **Shoichet, B. K., Baase, W. A., Kuroki, R., and Matthews, B. W.:** A relationship between protein stability and protein function, Proc. Natl. Acad. Sci. USA, **92**, 452–456 (1995).
39. **Kanaya, S., Oobatake, M., and Liu, Y.:** Thermal stability of *Escherichia coli* ribonuclease HI and its active site mutants in the presence and absence of the $Mg^{2+}$ ion: Proposal of a novel catalytic role for Glu48, J. Biol. Chem., **271**, 32729–32736 (1996).
40. **Beadle, B. M. and Shoichet, B. K.:** Structural bases of stability-function tradeoffs in enzymes, J. Mol. Biol., **321**, 285–296 (2002).
41. **Siddiqui, K. S.:** Defying the activity-stability trade-off in enzymes: taking advantage of entropy to enhance activity and thermostability, Crit. Rev. Biotechnol., **37**, 309–322 (2017).
42. **Rabia, L. A., Desai, A. A., Jhajj, H. S., and Tessier, P. M.:** Understanding and overcoming trade-offs between antibody affinity, specificity, stability and solubility, Biochem. Eng. J., **137**, 365–374 (2018).

43. **Tokuriki, N., Stricher, F., Serrano, L., and Tawfik, D. S.:** How protein stability and new functions trade off, PLoS Comput. Biol., **4**, e1000002 (2008).
44. **Tokuriki, N. and Tawfik, D. S.:** Chaperonin overexpression promotes genetic variation and enzyme evolution, Nature, **459**, 668–673 (2009).
45. **Bogumil, D. and Dagan, T.:** Cumulative impact of chaperone-mediated folding on genome evolution, Biochem, **51**, 9941–9953 (2012).
46. **Wyganowski, K. T., Kaltenbach, M., and Tokuriki, N.:** GroEL/ES buffering and compensatory mutations promote protein evolution by stabilizing folding intermediates, J. Mol. Biol., **425**, 3403–3414 (2013).
47. **Mack, K. L. and Shorter, J.:** Engineering and evolution of molecular chaperones and protein disaggregases with enhanced activity, Front. Mol. Biosci., **3**, 8 (2016).
48. **Agozzino, L. and Dill, K. A.:** Protein evolution speed depends on its stability and abundance and on chaperone concentrations, Proc. Natl. Acad. Sci. USA, **115**, 9092–9097 (2018).
49. **Giver, L., Gershenson, A., Freskgard, P. O., and Arnold, F. H.:** Directed evolution of a thermostable esterase, Proc. Natl. Acad. Sci. USA, **95**, 12809–12813 (1998).
50. **Xu, B. L., Dai, M., Chen, Y., Meng, D., Wang, Y., Fang, N., Tanga, X. F., and Tang, B.:** Improving the thermostability and activity of a thermophilic subtilase by incorporating structural elements of its psychrophilic counterpart, Appl. Environ. Microbiol., **81**, 6302–6313 (2015).
51. **Kim, E. J., Wu, C. H., Adams, M. W., and Zhang, Y. H. P.:** Exceptionally high rates of biological hydrogen production by biomimetic in vitro synthetic enzymatic pathways, Chem-A Euro. J., **22**, 16047–16051 (2016).
52. **Zhang, X. F., Yang, G. Y., Zhang, Y., Xie, Y., Withers, S. G., and Feng, Y.:** A general and efficient strategy for generating the stable enzymes, Sci. Rep., **6**, 33797 (2016).
53. **Li, G., Maria-Solano, M. A., Romero-Rivera, A., Osuna, S., and Reetz, M. T.:** Inducing high activity of a thermophilic enzyme at ambient temperatures by directed evolution, Chem. Commun. (Camb.), **53**, 9454–9457 (2017).
54. **Roth, T., Beer, B., Pick, A., and Sieber, V.:** Thermostabilization of the uronate dehydrogenase from *Agrobacterium tumefaciens* by semi-rational design, AMB Express, **7**, 103 (2017).
55. **Gao, H., Li, C., Bandikari, R., Liu, Z., Hu, N., and Yong, Q.:** A novel cold-adapted esterase from *Enterobacter cloacae*: characterization and improvement of its activity and thermostability via the site of Tyr193Cys, Microb. Cell Fact., **17**, 45 (2018).
56. **Huang, R., Chen, H., Zhou, W., Ma, C., and Zhang, Y. H. P.:** Engineering a thermostable highly active glucose 6-phosphate dehydrogenase and its application to hydrogen production in vitro, Appl. Microbiol. Biotechnol., **102**, 3203–3215 (2018).
57. **Zhao, H. Y. and Feng, H.:** Engineering *Bacillus pumilus* alkaline serine protease to increase its low-temperature proteolytic activity by directed evolution, BMC Biotechnol., **18**, 34 (2018).
58. **Tokuriki, N., Jackson, C. J., Afriat-Jurnou, L., Wyganowski, K. T., Tang, R., and Tawfik, D. S.:** Diminishing returns and tradeoffs constrain the laboratory optimization of an enzyme, Nat. Commun., **3**, 1257 (2012).
59. **Starr, T. N. and Thornton, J. W.:** Epistasis in protein evolution, Protein Sci., **25**, 1204–1218 (2016).
60. **Steinberg, B. and Ostermeier, M.:** Environmental changes bridge evolutionary valleys, Sci. Adv., **2**, e1500921 (2016).
61. **Arnold, F. H.:** Directed evolution: bringing new chemistry to life, Angew. Chem. Int. Ed., **57**, 4143–4148 (2018).