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Commentary

Enzyme engineering reaches the boiling point

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The boiled enzyme was toppled as a standard enzymology control when researchers in the 1970s started uncovering enzymes that loved the heat (1). Identification of a variety of intrinsically hyperstable enzymes from hyperthermophilic organisms, with optimal growth temperatures of 100°C and above, has piqued academic curiosity (e.g., how do these proteins withstand such "extreme" conditions?) and generated considerable interest for their possible applications in biotechnology (2, 3). The realization that enzymes can function at such high temperatures has spawned thermophily-envy, causing researchers and enzyme users to wonder whether their favorite mesophilic enzymes could be engineered to resist boiling, or at least long-term storage on a warm shelf. Perhaps their enzyme has no good thermophilic counterpart, or they do not relish tackling the sometimes considerable technical challenges of working with thermophiles or the enzymes they produce. Thus the literature is replete with testimonials to the power of mutagenesis for protein stabilization (4). With some notable exceptions (5, 6), the increases in stability have been less than impressive.

Van den Burg *et al.* (7), however, now have shown that a moderately thermostable thermolysin-like protease (TLP) can be made hyperstable, to the point that it functions at 100°C. The hyperstability is a result of substitution of only eight amino acids (out of 319). The engineered protease exhibits a 21°C increase in the temperature optimum for activity. Remarkably, this increased stability comes without reduction in the enzyme's proteolytic activity at 37°C.

It is thought that today's mesophiles all have evolved from a thermophilic ancestor, and therefore that extreme thermostability is a primitive rather than acquired characteristic (8). Thus perhaps we should be wondering why mesophilic enzymes are so unstable, rather than why thermophilic ones are so stable (9). Thermostability can be increased by the substitution of a small number of amino acids, but selection has not favored these sequences. Is reduced stability under direct selection pressure? Is there, for example, a critical balance between stability and lability that must be maintained for function (9, 10)? Or, alternatively, is reduced stability a natural consequence of selection for higher activity at lower temperatures (cold adaptation), because activating mutations tend to destabilize the protein? In other words, has thermostability simply drifted down while the enzymes adapt to activity in the cold? [If the latter is true, then engineering cold activity into a thermophilic enzyme may be easier than engineering thermostability into a cold active enzyme (8).] If enzymes instead are constrained by a very fine stability-lability balance, it should prove difficult, if not impossible, to stabilize a protein to a very high degree while maintaining its catalytic activity at low temperatures. Yet this is precisely what Van den Burg *et al.* (7) have done.

How was this remarkable increase in stability achieved? The short answer is with a lot of hard work! This group had shown previously that only a small subset of the 43 amino acid differences separating the TLP and its more-stable homolog, thermolysin, is responsible for their differing stabilities (11). The remaining mutations presumably are neutral or influence fitness in other ways, such as maintaining catalytic efficiency at physiologically useful levels. The 8-fold mutant was made by combining the five TLP-to-thermolysin mutations that were shown to enhance stability with three more "rationally designed" mutations (a Ser-to-Pro substitution believed to lower the entropy of the unfolded state and a disulfide bridge), also known to improve stability in the wild-type TLP background. The resulting enzyme is much more stable than thermolysin.

Those who wish to achieve a similarly impressive result are left with the dilemma of how to identify stabilizing amino acid substitutions. A thermophilic homolog often is not available. And even if there is one, determining which substitutions are stabilizing requires an heroic effort (11). Although thermophilic enzymes may strongly resemble their mesophilic counterparts in three-dimensional structure, their sequences differ considerably, usually much more than TLP and thermolysin, which share 85% sequence identity. Furthermore, testing single substitutions in the background of the mesophilic enzyme obviously does not probe for useful combinations of mutations. One can try to rationally design stabilizing mutations, but this is difficult, too. It is clear that a variety of (often subtle) stabilization mechanisms characterize natural thermophilic proteins and that the effects of mutations are context dependent. Unfortunately, the highly desired generally applicable rules by which a mesophilic protein could be made thermostable do not appear to exist.

We can take some comfort, however, in that few amino acid substitutions are needed to achieve useful stabilization. Even with a low success rate, a "rational" approach to stabilizing a protein still can provide useful results. But a far easier approach to making a mesophilic enzyme into a thermostable counterpart may be to direct its evolution in the laboratory, accumulating stabilizing mutations by random mutagenesis, recombination, and screening (or selection) (12, 13). The evolutionary design approach requires no structural information, knowledge of deactivation mechanisms, or understanding of the molecular basis for thermostability. Analysis of the molecular solutions obtained, however, may well contribute to this understanding.

How far can we go to increase protein thermostability? We still do not know what the upper limits of protein stability are, or even whether they are dictated by conformational stability or the degradation of amino acids (9). If the temperature limits to protein stability are less stringent than the temperature limits to life, however, it should be feasible to engineer enzymes even more thermostable than those found in nature.

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