Selection of mutations for increased protein stability

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There are many ways to select mutations that increase the stability of proteins, including rational design, functional screening of randomly generated mutant libraries, and comparison of naturally occurring homologous proteins. The protein engineer's toolbox is expanding and the number of successful examples of engineered protein stability is increasing. Still, the selection of thermostable mutations is not a standard process. Selection is complicated by lack of knowledge of the process that leads to thermal inactivation and by the fact that proteins employ a large variety of structural tricks to achieve stability.

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Introduction

Several factors that limit the industrial use of enzymes are related to the (lack of) stability of these biocatalysts. When developing enzymatic processes, conflicts between optimization of process conditions and the fragility of enzymes are likely to emerge. Thermal stability of the biocatalyst is of major importance as running a process at high temperatures has many advantages, for example, higher reaction rates, higher solubility of reactants and less microbial contamination. In general, better operational enzyme stability will reduce process costs.

During the past 15 years there has been a continuous flow of reports describing proteins stabilized by the introduction of mutations. These reports span a period from pioneering rational design work on small enzymes [1], to complete *in computo* design [2] and the use of random mutation strategies coupled with high-throughput screening for improved stability [3,4•,5•]. Along the way, many stabilizing mutations have been selected. Here we describe recent examples of mutant selection and discuss the current possibilities and limitations in engineering protein stability.

Lessons from proteins from extremophiles

One way to obtain hyperstable enzymes is to look for them in organisms that grow under extreme conditions. During the past decade, many such enzymes have been described and characterized in detail (recently reviewed in [6,7•]).

In principle, studies of naturally occurring hyperstable enzymes could provide important leads for the selection of stabilizing mutations. In its most simple form, this is indeed the case: there are numerous examples of proteins that have been stabilized by the introduction of residues or structural elements found in their homologous counterparts isolated from hyperthermophilic organisms [8–12]. Studies of hyperstable enzymes have, however, also shown that there is great variation in the (presumed) structural basis of hyperstability, meaning that it is difficult to derive general 'rules' for protein stabilization from the available data.

Two of the most prominent properties of naturally occurring hyperstable enzymes are the presence of large surface networks of electrostatic interactions and a tendency to be multimeric [6,7•,13]. There is abundant evidence in recent literature supporting the notion that these two properties indeed contribute to protein stability [13–16,17•,18,19]. Unfortunately, these properties are not easy to engineer into any protein one wants to stabilize.

Electrostatic networks are complex and involve cooperative interactions between several residues. The findings in hyperstable enzymes have revived interest in the contribution of charged surface residues to protein stability [20–22]. This has yielded some interesting studies, clearly showing that surface electrostatics is worth looking at when stabilizing a protein [23–25,26•,27]; however, there are hardly any examples of proteins that have been significantly stabilized by engineering designed networks of electrostatic interactions on the surface.

Multimerization can be engineered in the sense that one may stabilize a multimeric protein by strengthening the (often electrostatic) interactions between the monomers [15,16,19]. However, converting a monomeric protein into a stable multimer is not a simple task and cannot be reckoned as an 'obvious' strategy for the protein stability engineer.

Ever since the early days of stability engineering, one has sought after statistical 'rules' that would enable the stabilization of any protein, possibly even without the availability of a crystal structure. It is now more clear than ever that finding such rules makes limited sense. Every single mutation needs to be evaluated in its specific structural context, while taking into account the character of the thermal inactivation mechanism (see below) of the protein in question [7•,28•,29•]. Work on extremophilic enzymes certainly has yielded new clues as to how one might try to achieve protein stabilization by mutagenesis, but has not revealed a defined and secure road to success.

It is important to note that there are many examples in the literature of proteins that have been stabilized dramatically by just a very limited number of mutations [10,12,30,31]. In fact, theoretically, this is to be expected as the net

stability of a protein equals the energy of only a very limited number of beneficial interactions. Although this observation in principle enlightens the task of the protein engineer (only a few mutations are needed), it underlines the fact that sequence statistics are not of much use.

The consensus approach

The consensus approach is based on the assumption that conserved residues, as inferred from an alignment of homologous amino acid sequences, contribute more to stability than residues that are not conserved. Indeed, it has been shown that highly stable fungal phytases may be obtained by combining 'consensus' residues in one multiple mutated variant [32]. These rather surprising results may be rationalized in part by assuming a common thermophilic ancestor. This idea was recently corroborated by Miyazaki *et al.* [33•], who used phylogeny to identify ancestral residues in 3-isopropylmalate dehydrogenases. It was shown that natural variants of the enzyme lacking some of the ancestral residues could be stabilized by introducing these residues. The consensus approach has recently been used to study the importance and evolution of packing in the hydrophobic core of staphylococcal nuclease, which contributes to the stability of the enzyme [34]. Of the many sequences tested, the consensus sequences were among the better ones, but were not necessarily the best. In conclusion, it seems that the consensus approach may be a valuable additional tool for the protein stability engineer, which exploits the vast amount of protein sequences that are available today.

Lessons from random mutagenesis studies

The recent development of different molecular breeding technologies (e.g. directed evolution, error-prone polymerase chain reaction [PCR] and gene shuffling) has led to the creation of many new biocatalysts, including variants with significantly improved stability characteristics (for recent reviews see [5•,35,36,37•]). The potential of these methods for stability engineering is illustrated by the construction of subtilisin variants with an up to 1200 times increase in $t_{1/2}$ at 60°C [38,39], an esterase showing a 14°C increase in T_m [3], a galactose oxidase with a 4°C increase in T_{50} [40] and a cold-shock protein with a 28°C increase in T_m [4]. Analysis of the selected amino acid substitutions has not provided straightforward explanations concerning the molecular basis of the stabilization that was obtained [41]. In general, the mutations selected by random mutagenesis approaches are scattered over the protein molecule (often near the surface) and their effects cannot always be explained on the basis of generally accepted principles of protein stability.

The screening procedures that are employed in approaches based on random mutagenesis need major attention. The screening procedure must be both suitable for high-throughput analysis and sufficiently discriminative. Preferably, assay conditions are such that only stabilized variants are active, thus enabling them to be detected in a single step. Several new approaches are being developed to accomplish further improvements in high-throughput screening for stability [42].

Among the screening procedures for thermostable biocatalysts, exploitation of thermophilic hosts could be a promising alternative. Unfortunately, to date, the expression of and selection for (essential) gene products in such hosts has not been explored to a great extent. Partly, this is because of the poor genetic accessibility of thermophiles. At present, the number of genetic tools available for thermophilic hosts is still limited. Nevertheless, cloning and selection in the thermophilic bacterium *Thermus thermophilus* was recently exploited successfully for the selection of thermostable variants of 3-isopropylmalate dehydrogenase from *Saccharomyces cerevisiae* [43].

Rational engineering and design

As described above, neither studies of proteins from thermophilic organisms nor efforts in various types of protein engineering have yielded a simple set of 'rules' for protein stabilization with general practical application. Nevertheless, several rational approaches for protein stabilization do exist and these have been applied in successful stability engineering of a variety of proteins. These approaches include improvement of the packing of the hydrophobic core, the introduction of disulfide bridges, stabilization of α-helix dipoles, engineering of surface salt bridges, and point mutations aimed at reducing the entropy of the unfolded state (Xaa→Pro and Gly→Xaa mutations, where Xaa is any amino acid; reviewed in [6,7•] and in many reviews published in the previous decade). Each of these approaches has been shown to work in some cases, but many failures do exist too (most of which do not appear in the literature).

Entropic stabilization is one of the most promising strategies for the protein engineer (for some recent examples see [44–46]) and is also employed by nature [8,47]. A prerequisite for mutations that decrease the entropy of the unfolded state is that the mutations do not introduce unfavorable strain in the folded protein structure. Thus, careful examination of the available structural information is necessary. Nevertheless, from a modelling point of view, entropic stabilizations (especially Ala→Pro and Gly→Ala) are among the ones that are most easy to evaluate. The main challenge is to assess the occurrence and possible effects of steric overlap that may be introduced (e.g. [9]).

One of the most important developments in stability engineering over the past few years has been the revived interest in the protein surface and in surface salt bridges. For a long time the protein surface was considered relatively uninteresting, because surface mutations were supposed to affect protein stability only marginally. In addition, some ten years ago, there was a certain consensus saying that surface salt bridges were not that important for stability. Remarkably, however, many of the recent stability

engineering studies mentioned above concern surface-located mutations, in several cases affecting surface electrostatics [4•,23,24,26,38,44,48].

Probably one of the best analyses of the importance of protein surfaces and surface electrostatics for stability comes from recent studies on the cold-shock proteins CspB and CspC from *Bacillus subtilis* and *Bacillus caldolyticus*, respectively [4•,10,11,26•]. These and other studies [23] emphasize that one should not only pay attention to salt bridges, but also to the overall charge distribution and to longer-range electrostatic interactions. It is important to note that surface salt bridges presumably become more favorable for stability as temperature increases [20,21].

Surface residues are particularly important in proteins whose thermal inactivation is governed by local (as opposed to global) unfolding processes (see below). Obviously, such local (partial) processes are expected to mostly involve the protein surface.

Unfortunately, the rational design of mutations affecting the protein surface and/or electrostatics is intrinsically difficult. The surface of the protein is more flexible than other parts and its structure is usually less well defined (high B factors). In addition, the surface structure observed in X-ray crystal structures may in part be the result of (artificial) crystal contacts. Thus, the structural finesse of the protein surface is difficult to assess in 'static' modeling programs. Electrostatic calculations on proteins are still extremely difficult and, consequently, the prediction of mutations that could improve stability by means of electrostatic interactions is complicated [22]. One advantage of directing mutagenesis efforts to the protein surface is the high chance of obtaining an active form of the protein. In general, structural adaptability is much larger in surface regions than in the core of the protein. Thus, the chances of completely ruining the protein structure are small for a surface mutation, but much larger for a core mutation.

Pitfalls in rational design

One major advantage of engineering stability by the random approach is that one does not need to know much about the thermal inactivation process. The success of rational design is, however, greatly dependent on sufficient knowledge of the inactivation mechanism. If one is sure that stability of the protein in question depends on (reversible) global unfolding, stability is relatively easy to rationalize. In such cases, it makes sense to apply the well-understood mutational strategies described above (e.g. entropic stabilization). By studying the structure and using modeling programs such as WHAT IF [49], one can design stabilizing mutations anywhere in the protein and one may expect a reasonable success rate.

In most cases, and especially under industrial conditions, thermal inactivation is not likely to depend on global unfolding, but rather on partial (local) unfolding followed by an irreversible process such as aggregation or proteolysis [28•,50–53]. This has profound consequences for stability engineering as the effects of a mutation will no longer only depend on its thermodynamic effect, but also on its location in the protein. Consequently, not every mutation that is thermodynamically stabilizing will indeed stabilize against irreversible thermal inactivation. Likewise, the best mutations for kinetic stability are not necessarily the best ones for thermodynamic stability [54]. The literature contains a few studies showing discrepancies between mutational effects on thermodynamic and kinetic stability [51,54,55]. The more local the stability-determining unfolding process is, the larger the chance that a designed stabilizing mutation simply does not affect kinetic stability at all. We have seen many such mutations in our own laboratories (in various proteins). Obviously, such mutations are not likely to appear in the scientific literature.

Our own work on engineering the stability of *Bacillus* neutral proteases has underlined the importance of local unfolding for stability [28•,44,56]. Of the very many potentially stabilizing mutations that were made, only a few were clearly stabilizing and these were all clustered in a certain surface region of the protein. This region is crucial in a local unfolding process that is rate-limiting for thermal inactivation (irreversibility is in this case caused by autoproteolysis). Once this region had been identified, stabilization of the enzyme by rational design proved to be relatively straightforward [30,44]. Proteases such as these are likely to be quite special in that they have very high kinetic stabilities, while being thermodynamically moderately stable or even unstable. Recent studies of the folding and stability of α-lytic protease provide fascinating insight into the peculiarities of the stability of certain proteases. These studies show the necessity of considering protein folding energetics and dynamics when setting up stabilization strategies [57•].

Stabilizing mutations and activity

Natural hyperstable enzymes tend to have low activity at medium temperatures. For a long time, it was thus believed that high stability could only be achieved by sacrificing activity at low temperatures. It is now clear that this is not the case. As long as stabilizing mutations do not have direct detrimental effects on active-site residues or active-site architecture, it is fully possible to engineer hyperstable enzymes that retain their activity at low temperatures [5•,31,44].

Conclusions and perspectives

In recent years the protein engineer's knowledge-base and toolbox for the prediction and introduction of thermostability-improving mutations have changed quite considerably. The advent of new stabilization strategies based on random mutagenesis coupled to high-throughput screening probably represents the most important development. With respect to the development of our knowledge-base, two developments stand out. One is the increasing

awareness of the importance of the protein surface for stability. In the early days of protein engineering much stability-related research was focussed on stabilization of the hydrophobic core and other more or less 'internal' structural elements (see [58] for an interesting and early exception). Recent research, however, has unambiguously shown that the surface of the protein should be one of the first targets for a protein stability engineer.

A second important development in rational stability engineering is the increased understanding of how the character of the thermal inactivation mechanism affects the success of a stabilization strategy. The choice of a rational stabilization strategy for a biocatalyst must depend on the envisioned application of the biocatalyst, as process conditions may affect the thermal inactivation mechanism.

Although nowadays several powerful strategies for protein stabilization are available to the protein engineer, an easy-to-use high-precision tool set for rational stability engineering is still lacking. The accumulated information from genome sequencing projects and from the increasing number of structures (both X-ray and NMR) of psychrophilic, mesophilic, thermophilic and hyperthermophilic homologous proteins indicates that mechanisms underlying thermostability are highly diverse. However, in view of the anticipated forthcoming increase in available data, from rational mutagenesis, directed evolution, genome sequencing and structure determinations, it is likely that the tool set will become more user friendly and accurate in due time.

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