



## Rational engineering of enzyme stability

Vincent G.H. Eijssink<sup>a,\*</sup>, Alexandra Bjørk<sup>b</sup>, Sigrid Gåseidnes<sup>a</sup>, Reidun Sirevåg<sup>b</sup>,  
Bjørnar Synstad<sup>a</sup>, Bertus van den Burg<sup>c</sup>, Gert Vriend<sup>d</sup>

<sup>a</sup> Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway, P.O. Box 5040, N-1432 Ås, Norway

<sup>b</sup> Department of Molecular Biosciences, University of Oslo, P.O. Box 1041 Blindern, N-0316 Oslo, Norway

<sup>c</sup> IMEnz Bioengineering, Kerklaan 30, P.O. Box 14, 9750 AA Haren, The Netherlands

<sup>d</sup> Center for Molecular and Biomolecular Bioinformatics, University of Nijmegen, P.O. Box 9010, 6500 GL Nijmegen, The Netherlands

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### Abstract

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 such as T4 lysozyme and barnase to protein design, and directed evolution. Concomitantly, the purification and characterization of naturally occurring hyperstable proteins has added to our understanding of protein stability. Along the way, many strategies for rational protein stabilization have been proposed, some of which (e.g. entropic stabilization by introduction of prolines or disulfide bridges) have reasonable success rates. On the other hand, comparative studies and efforts in directed evolution have revealed that there are many mutational strategies that lead to high stability, some of which are not easy to define and rationalize. Recent developments in the field include increasing awareness of the importance of the protein surface for stability, as well as the notion that normally a very limited number of mutations can yield a large increase in stability. Another development concerns the notion that there is a fundamental difference between the “laboratory stability” of small pure proteins that unfold reversibly and completely at high temperatures and “industrial stability”, which is usually governed by partial unfolding processes followed by some kind of irreversible inactivation process (e.g. aggregation). Provided that one has sufficient knowledge of the mechanism of thermal inactivation, successful and efficient rational stabilization of enzymes can be achieved.

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### 1. Introduction

Stability is an important parameter, which co-determines the economic feasibility of applying an enzyme in an industrial process. High stability is gen-

\* Corresponding author. Tel.: + 47-64949472;  
fax: +47-64947720.

E-mail address: [vincent.eijssink@ikbm.nlh.no](mailto:vincent.eijssink@ikbm.nlh.no) (V.G.H. Eijssink).

erally considered an economic advantage because of reduced enzyme turnover. In addition, stable enzymes permit the use of high process temperatures, which may have beneficial effects on reaction rates, reactant solubility and the risk of microbial contamination. Enzymes are increasingly used in feed where they may need to withstand hygienic heat treatments and harsh processes such as extrusion.

Since the beginning of large scale (recombinant) enzyme production for industrial applications, protein engineering methods have been applied to improve enzyme properties. The best-known example is the work on subtilisin (Bryan, 2000) and closely related proteases, which are widely used in detergents. Studies on the stability of small enzymes which unfold reversibly at high temperatures have permitted thermodynamic assessment of certain types of interactions for protein stability (e.g. Matthews, 1993). This has led to the identification of several general strategies for protein stabilization, e.g. “entropic stabilization” (rigidification) by Gly → Ala, Xxx → Pro mutations or the introduction of disulfide bridges (Matthews et al., 1987; Matsumura et al., 1989; Clarke and Fersht, 1993; Mansfeld et al., 1997), “helix capping” by introducing residues that interact with the alpha-helix dipole (e.g. Nicholson et al., 1988, 1991; Sali et al., 1988; Serrano and Fersht, 1989; Marshall et al., 2002), other types of helix optimisation (Serrano et al., 1992; Blaber et al., 1993), the introduction of salt bridges (Serrano et al., 1990; Dao-Pin et al., 1991; Waldburger et al., 1995; Strop and Mayo, 2000; Pace et al., 2000; Makhatadze et al., 2003; Schwehm et al., 2003), and the introduction of clusters of aromatic–aromatic interactions (Burley and Petsko, 1985; Serrano et al., 1991; Anderson et al., 1993; Puchkaev et al., 2003).

In the past decade, large numbers of enzymes isolated from extremophiles have been studied and compared to their counterparts from mesophilic sources. The conclusion from a large collection of such comparative studies, only few of which are substantiated by mutagenesis work, is that nature has employed many different structural strategies for obtaining high stability (reviewed by Sterner and Liebl, 2001; Vieille and Zeikus, 2001; and by many others, see e.g. Hakulinen et al., 2003 for a recent study). The notion that high stability may be achieved by many different “structural routes” is confirmed by the results of directed evolution studies of protein stability (Giver et al., 1998;

Zhao and Arnold, 1999; Spiller et al., 1999; Hoseki et al., 1999; Miyazaki et al., 2000; Wintrode and Arnold, 2000; Martin et al., 2001; Arnold et al., 2001). These recent developments have revealed some important trends (Van den Burg and Eijsink, 2002): (1) the surface and surface electrostatics of a protein are more important for stability than previously thought (Eijsink et al., 1995; Hoseki et al., 1999; Perl et al., 2000; Martin et al., 2001, 2002; Perl and Schmid, 2001; Machius et al., 2003); (2) large networks of electrostatic interactions and a higher oligomerization state are presumably favourable for stability, since these features are frequently observed in enzymes from extremophilic sources (Vetriani et al., 1998; Xiao and Honig, 1999; Thoma et al., 2000; Clantin et al., 2001; Vieille and Zeikus, 2001; Walden et al., 2001; Maeda et al., 2002).

There are now several examples of proteins which have been stabilized by the introduction of numerous mutations with cumulative small stabilizing effects (e.g. Zhang et al., 1995; Giver et al., 1998; Wintrode and Arnold, 2000; Lehmann et al., 2002; D’Amico et al., 2003a), which may be taken to suggest that it is useful to use sequence statistics or statistical structural comparisons to discover “rules” for protein stability. However, a clear conclusion to be drawn from other recent work is that very large stability differences in some cases are due to only one or very few point mutations (e.g. Williams et al., 1999; Hasegawa et al., 1999; Sandgren et al., 2003). It has also been shown that stability differences between homologous enzymes may be due to very few (out of many) of the naturally occurring sequence variations (Serrano et al., 1993; Veltman et al., 1996; Sandgren et al., 2003). Obviously, these latter observations indicate that statistical comparisons of the sequences and structures of proteins with varying stabilities may not be that useful.

The stabilities of proteins that unfold completely and reversibly under denaturing conditions may be assessed by equilibrium measurements, permitting quantification of mutational effects in terms of changes in the  $\Delta G$  of folding (e.g. Pace, 1990). Many proteins do not unfold reversibly and if they do so in the laboratory, they do not necessarily behave likewise in the more complex environment of an enzyme reactor. Therefore, for most industrial enzymes, the only stability parameters that are relevant and assessable relate to kinetic (and not thermodynamic) stability.

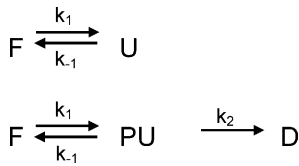


Fig. 1. Thermal unfolding and denaturation. F, folded protein; U, reversibly unfolded protein; PU, partially unfolded protein; D, denatured protein (aggregated, degraded). The upper equation applies to thermodynamic stability; the lower equation applies to kinetic stability. Irreversible thermal inactivation often follows first-order kinetics, indicating that, in the lower equation,  $k_1$  is limiting the overall rate of the thermal inactivation process (e.g. Eijnsink et al., 1991; Vriend and Eijnsink, 1993; Bryan, 2000; Machius et al., 2003).

Irreversible thermal denaturation of a protein usually comprises an unfolding step followed by an irreversible process (aggregation or, in some cases, proteolysis; Fig. 1). Studies on protein aggregation and proteolysis strongly suggest that the unfolding steps that make a protein amenable to these processes most often have a partial/local (as opposed to global) character (Eijnsink et al., 1992a; Vriend and Eijnsink, 1993; Fink, 1998; Finke et al., 2000; see also recent literature on amyloid formation, e.g. Chiti et al., 2002; Thirumilai et al., 2003; Doyle et al., 2003). It has been pointed out that such partial unfolding processes primarily involve surface-located parts of the protein, explaining why the protein surface has been found to be important for kinetic stability in several cases. When designing mutations for stabilization against irreversible processes, one may use the same reasoning as for stabilization against reversible, global unfolding, but with two important differences. First, a mutation that increases the  $\Delta G$  of reversible global unfolding will only affect kinetic stability if it affects the (local) stability of the regions whose unfolding triggers the irreversible inactivation (Eijnsink et al., 1992b). For example, to protect an enzyme against proteolytic cleavage, mutations must stabilise the region of the protein whose unfolding is necessary for the first, rate-limiting cleavage to occur. Second, mutations that are thermodynamically neutral or unfavourable can stabilize a protein against irreversible denaturation because of an effect on unfolding kinetics and local flexibility. This possibility has been discussed in literature, but so far there is little experimental support (Chrnyk and Wetzel, 1993; Clarke et al., 1995; Dürschmidt et al., 2001).

We discuss some aspects of rational design of stabilizing mutations. In addition, we will illustrate the

possibilities and pitfalls of rational engineering of protein kinetic stability by reviewing our own work on a variety of enzymes and by alluding to some interesting recent reports in the literature.

## 2. Rational design of stabilizing mutations

The rational design of a mutation that will increase the  $\Delta G$  of folding requires a deep understanding of the forces underlying the energy balance of protein and solvent in the folded as well as the unfolded state.  $\Delta G = \Delta H - T\Delta S$  indicates that there are principally two ways to stabilize a protein, via  $\Delta H$  or  $\Delta S$ . In practice it is nearly impossible to decompose the effect of a mutation in  $\Delta\Delta H$  or via  $\Delta\Delta S$  terms by reasoning, but it is common practice to do so anyway. For example, a Ser  $\rightarrow$  Pro mutation in a surface loop is not likely to remove or add any nice atomic interactions ( $\Delta\Delta H$ ). This mutation is more likely to affect  $\Delta G$  by reducing the entropy of the unfolded protein and is therefore, often referred to as an example of “entropic stabilization” (Matthews et al., 1987). If the  $\Delta\Delta H$  and  $\Delta\Delta S$  of such a mutation are measured, however, it will be seen that enthalpy does play a role, showing the dangers of over-simplification.

It is obvious that all terms that contribute to  $\Delta H$  and/or  $\Delta S$  contribute to  $\Delta G$  and thus, to the stability of a protein. Some important terms are Van der Waals’ interactions, hydrogen bonds, salt bridges, torsion potentials, bond stretching, planarity of conjugated systems, pi-pi stacking, the entropy of water (this is by far the biggest term of all, and probably the least well understood), interactions with ions, loop tension, helix dipole interactions, and disulfide bridges. In case of stability against reversible, global unfolding all these terms contribute and it is not possible to assign the stability to one particular term. Literature shows a continuous stream of articles with titles like “salt bridges are important for the stability of ...”, “water exclusion determines the stability of ...”, etc. It is important to underline that such papers describe examples that worked well, but do not provide unique, generally applicable routes to stability. Neither does a collection of these papers necessarily provide a comprehensive collection of “all” roads to stability: each protein can be made more stable against reversible, global unfolding by affecting each of the terms mentioned above.

The forces underlying the stability of a protein are the main topic of molecular dynamics and energy minimisation studies on proteins, and the results of research in this field are directly applicable to predicting the stabilising effect of point mutations. Unfortunately, the forces that govern protein folding and structure are not fully understood, and much research in this direction is still needed. For example, none of the commonly available software packages deals with complexities like induced charges, multiple protonation states, multibody interactions, and multipole interactions. Despite many developments in this field, fundamental improvements are needed before the interactions that govern folding and structure of a protein can be properly calculated. The two major routes around this problem are computational tricks that will cause most unknown terms to cancel out (thermodynamic cycles), and the use of statistically determined force fields (Krieger et al., 2002).

Statistical force fields are used in many design and modelling procedures, varying from the design of point mutations to drug docking and the design or homology modelling of complete proteins. Although there are no principal differences in force field requirements between these procedures, the use of force fields based on just statistics is most useful for the simplest of these procedures, namely design of single point mutations. Such methods for mutant design are all based on some fitness parameter. We routinely use directional contact analysis (Vriend and Sander, 1993), but many more energy-emulating scoring functions exist. All these methods look for aspects of a protein structure that can be tabulated and expressed as preference parameters. Such aspects can be torsion angles, atomic contacts, hydrogen bonds, solvation parameters, excluded solvent, etc. In most mutation prediction methods the simple, but effective, rule that a mutation leaves the rest of the protein intact (De Filippis et al., 1994) is applied and all 20 residue types are tried at one position. We use position specific rotamers (Chinea et al., 1995) extracted from the WHAT IF (Vriend, 1990) database, but other methods exist (Ponder and Richards, 1987; Holm and Sander, 1991; Cardozo et al., 1995). The WHAT IF mutation predictor is freely available at <http://www.cmbi.kun.nl/gv/servers/WIWWWI/>. Similar technology exists for the prediction of disulfide bridges (Hazes and Dijkstra, 1988), and is available from the same server.

The design of single point mutations may be taken all the way to automated computational redesign of complete proteins (Dahiyat and Mayo, 1997; Malakauskas and Mayo, 1998; Hellinga, 1998; Pokala and Handel, 2001; Filikov et al., 2002; Ventura et al., 2002; Ewert et al., 2003; Mooers et al., 2003; Dantas et al., 2003). Such redesign is based on the “inverse folding approach”, meaning that one tries to optimise the sequence for a given structure. Several groups have developed effective methods to handle the combinatorial complexity connected to protein redesign, while using force fields based on either statistics or first principles of protein folding and stability, or hybrids of these two. This has led to the production of several redesigned proteins with interesting properties (e.g. Malakauskas and Mayo, 1998; Filikov et al., 2002; Ventura et al., 2002; Mooers et al., 2003; Dantas et al., 2003). Malakauskas and Mayo (1998) described the design and construction of a hyperthermostable variant of the 55 residue B1 domain of streptococcal protein G, a landmark in fully automated design of stable proteins.

### 3. Examples of engineered kinetic stability

#### 3.1. Thermolysin-like proteases

Bacteria belonging to the genus *Bacillus* produce a zinc metalloprotease, called neutral protease or thermolysin-like protease (TLP; after the best-known member of this family of homologous enzymes crystallized in 1972; Matthews et al., 1972). Thermal inactivation of TLPs follows first-order kinetics and is governed by a rate-limiting unfolding step that precedes the irreversible process of (rather complete) autolysis (Eijsink et al., 1991; Vriend and Eijsink, 1993; Vriend et al., 1998; Kidokoro et al., 1995; see also Zhao and Arnold, 1999). Naturally occurring TLPs exhibit large differences in thermal stability (Veltman et al., 1997a), making these enzymes interesting subjects for the study and engineering of protein stability (Imanaka et al., 1986; Vriend and Eijsink, 1993; Eijsink et al., 2001).

Much insight into the stability of TLPs has come from protein engineering studies of the TLP from *Bacillus stearothermophilus* CU21 (TLP-ste; Takagi et al., 1985), and from the comparison of this enzyme with its more thermostable counterpart thermolysin. The ki-

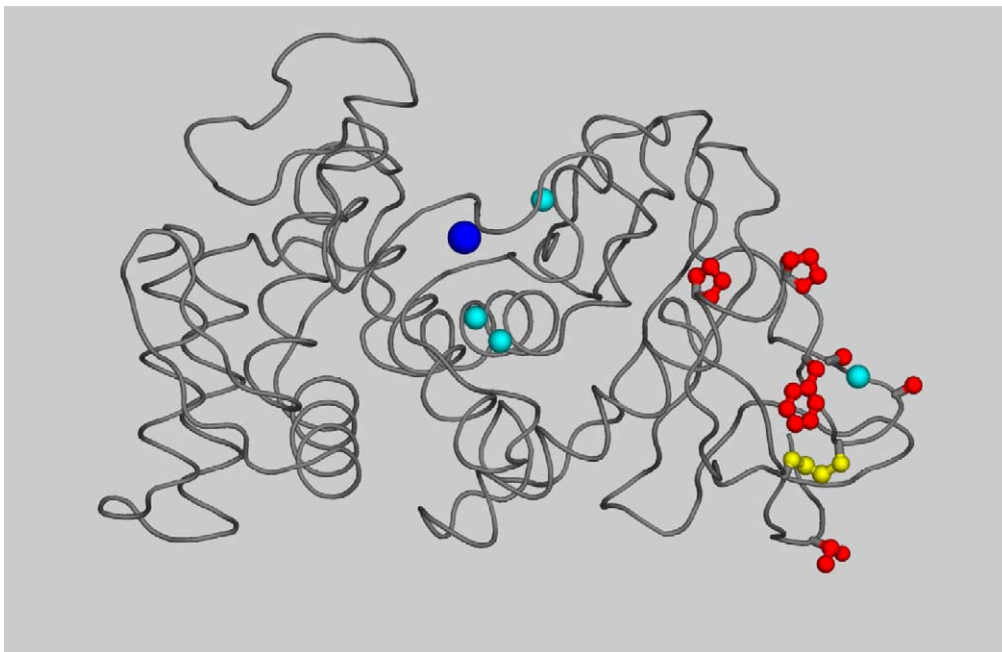


Fig. 2. Overview of the hyperstable eight-fold mutant of TLP-ste (“boilylsin”). The picture shows a backbone trace of TLP-ste. The side chains of the eight residues that were introduced by mutation are shown in red (Thr4, Ala56, Ala58, Phe63, Pro65, Pro69) or yellow (Cys8 and Cys60) and delineate the identified partial unfolding region or “weak spot”. The dark blue and light blue balls indicate bound zinc and calcium ions, respectively (the zinc ion is located in the catalytic center). The C-terminal domain is located left of the zinc atom, whereas the N-terminus and N-terminal domain are located to the right. The structure of TLP-ste was modelled by homology on the basis of the thermolysin structure, while omitting the three-residue insertion. The backbone trace shown here is identical to that of thermolysin (Matthews et al., 1972). All pictures of protein structures were made using PyMOL (DeLano, 2002).

netic thermal stability of TLPs may be expressed as  $T_{50}$ , which is the temperature required to reduce initial activity by 50% in 30 min (Eijsink et al., 1991). TLP-ste differs at 44 of its 319 positions from the 316-residue thermolysin (including a three residue insertion) and displays a 13.5 °C lower  $T_{50}$  ( $T_{50}$ 's are 86.9 °C and 73.4 °C for thermolysin and TLP-ste, respectively). TLP-ste has been subjected to extensive mutagenesis studies with the following major results:

- The mutational effects of various types of designed mutations in the C-terminal domain (which coincidentally had been selected for these studies) were almost always in the +1 to –1 °C range (summarized by Eijsink et al., 1995).
- Systematic replacement of residues in TLP-ste by the corresponding residue in thermolysin (Van den Burg et al., 1991; Veltman et al., 1996) yielded several mutants displaying relatively large stabilizing effects (varying from –6.3 to + 7.0 °C). Muta-

tions with large effects were clustered in a surface-located region in the N-terminal domain of the protein (Fig. 2). This region thus seems to be a “weak spot” or “unfolding nucleus”.

- Further mutagenesis of this region showed that single point mutations could have dramatic effects on stability (from –23.0 to + 4.7 °C for designed single point mutations and upto + 16.7 °C for the introduction of a disulfide bridge; Veltman et al., 1997a; Mansfeld et al., 1997). So, once the weak spot was known, most designed mutations were successful and had considerable stability effects.

These studies clearly suggest that the thermal inactivation of TLP-ste is governed by an unfolding process in the N-terminal domain. This notion was further supported by the results of combining stabilizing mutations identified in the comparative study. A TLP-ste variant containing only two of these mutations (T63F,

A69P) was 12.3 °C more stable than the wild-type (that is only 1.2 °C less stable than thermolysin), whereas adding three more “natural” mutations (A4T, T56A, G58A) increased the  $\Delta T_{50}$  to + 19.9 °C, yielding an enzyme that was considerably more stable than thermolysin itself (Eijssink et al., 1995). Combination of these five “natural” mutations with three designed mutations (S65P and a disulfide obtained via introducing G8C and N60C) yielded an enzyme with a  $T_{50}$  of 102 °C, that is 26.8 °C higher than wild-type TLP-ste. The half-life of this eight-fold mutant at 100 °C was 170 min, whereas TLP-ste had a half-life of 1.5 min at 90 °C (Van den Burg et al., 1998, 1999; Arnold, 1998). This TLP-ste variant (Fig. 2), which has been called boilylsin (Van den Burg et al., 1999), was and still is one of the most stable enzymes ever obtained by protein engineering.

The effects of site-directed mutations of thermodynamic stability are expected to be additive as long as the mutated residues do not interact (Wells, 1990; Zhang et al., 1995). However, when thermal inactivation is governed by partial unfolding processes, non-additivity is expected since the effects of mutations in one region of the protein depend on how much unfolding in this region contributes to the overall thermal inactivation process. Thus, the effect of a mutation in one region depends on how stable other regions of the protein are. Indeed, clear-cut cases of non-additivity were observed for TLP-ste. For example, a deleterious double mutation in the hydrophobic core of the C-terminal domain (L284W, F310W) yielded a  $\Delta T_{50}$  of only  $-0.9$  °C when introduced in the wild-type enzyme, whereas it destabilized the 23.5 °C more sta-

ble A4T-T56A-G58A-T63F-S65P-A69P mutant by as much as 7.1 °C. So, the effect of deleterious mutations in the C-terminal domain became more noticeable in a situation where the N-terminal domain had been stabilized (see Vriend et al., 1998, for further discussion).

Five of the eight mutations in boilylsin are of the “entropic stabilization”/rigidification type (G58A, S65P, A69P, G8C-N60C), confirming the power of this type of mutations for protein stabilization. It is probably better to refer to these mutations as “rigidifying”, since the “entropic” effect primarily applies to a state (the globally unfolded state) that is never reached during irreversible thermal inactivation of TLPs. The work on TLP-ste also led to discovery of a new type of stabilizing mutation: residue 63 is located at the protein surface (Fig. 3), meaning that the relatively large stabilizing effect of replacing Thr by Phe (Van den Burg et al., 1991) came as a surprise. Analysis of a series of mutations at position 63 yielded the conclusion that, apparently, hydrophobic interactions between the phenylalanine ring and aliphatic parts of the surrounding (mostly polar) side chains were beneficial for stability (Van den Burg et al., 1994). Interestingly, a few similar examples have appeared in the literature in recent years (Hillier et al., 1998; Schindler et al., 1998; Schwehm et al., 1998; Machius et al., 2003; see also Tisi and Evans, 1995; Ceruso et al., 1999; Gromiha et al., 2002), emphasizing that the protein surface and a variety of interactions at this surface are important for stability. There are indications that near-surface hydrophobic clusters may be particularly useful in beta-strand structures (such as in the TLP case; Fig. 3), since such clusters effectively strengthen interactions between adjacent beta-strands

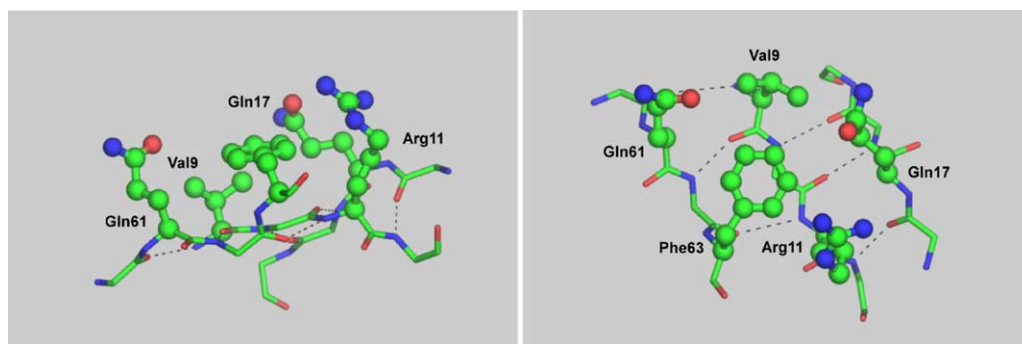


Fig. 3. Two views of the environment of Phe63 in thermolysin. All side chains shown, except residue 63, are conserved between thermolysin and TLP-ste. The dashed black lines indicate hydrogen bonds.

(Fig. 3; Tisi and Evans, 1995; Ceruso et al., 1999; Espinosa et al., 2001).

Thermolysin and TLP-ste bind four calcium ions, one of which is located in the stability-determining region (Fig. 2). It has been shown that the calcium-dependency of stability under the conditions used for standard stability assays reflects binding to the Ca3 site, that is the site in the unfolding region (Veltman et al., 1997b, 1998). It has also been shown that the destabilizing effect of mutations which deteriorate this calcium site can be compensated by stabilizing mutations near the binding site (Veltman et al., 1997b, 1998). Thus, in TLPs calcium binding is one of several mechanisms to create local stability in the unfolding region.

Subtilisin is a broad-specificity protease which resembles TLPs in the sense that it becomes thermally inactivated as a consequence of local unfolding followed by autolysis (Zhao and Arnold, 1999; Bryan, 2000). Another similarity concerns the presence of weak and strong binding sites for calcium and the calcium-dependency of thermal stability. Literature contains reports on hundreds of stability mutants in subtilisins. Taken together (Bryan, 2000) the results of these studies show quite some similarity with the results for TLPs. For example, almost all mutations that improve stability concern surface-located residues, and many of the mutations that affect stability somehow seem to affect a weak calcium-binding site. In a recent directed evolution study (Miyazaki et al., 2000), all mutations in a selected stable seven-fold mutant of a psychrophilic subtilisin were located on the surface and in one half of the molecule, most of them close to a weak calcium-binding site.

### 3.2. Chitinase from *Serratia marcescens*

Mutational strategies such as replacement of glycines and the introduction of prolines (entropic stabilization; Matthews et al., 1987) are used relatively often with quite some success (Serrano et al., 1992; Hardy et al., 1993; Watanabe et al., 1994; Bogin et al., 1998). In a recent study, we applied this strategy to a 499 residue two-domain chitinase, which unfolds and aggregates irreversibly at elevated temperatures. We used a semi-automated mutant design procedure in WHAT IF (Vriend, 1990), which uses a statistical force field to find positions in a protein where Ala or Pro fit well and where the natural residue does not seem to be

optimal. On the basis of our previous experience with the TLPs and emerging ideas about the partial character of the unfolding processes governing irreversible thermal inactivation, we restricted mutant design to positions at or close to the surface.

Fifteen mutants were made, which were all expressed and had wild-type like activity. Interestingly, many of these mutants showed only marginal stability effects. There were only three exceptions and these were clustered in or very close to a long surface meander (Fig. 4). On the basis of these observations, one might speculate that the surface meander highlighted in Fig. 4 plays a role in the early steps of chitinase unfolding (Gåseidnes et al., 2003).

The results of these fifteen semi-automatically designed mutations illustrate that it is rather straightforward to predict acceptable and potentially beneficial mutations. At the same time, the results suggest (as for the TLPs) that mutations only work against irreversible processes if they are introduced in certain crucial regions of the protein. One might argue that finding these regions is more complicated than designing stabilizing mutations once these regions are found.

### 3.3. Tetrameric malate dehydrogenases

Unfolding and thermal denaturation of multimeric enzymes is not easy to analyze and rationalize in detail, because it is difficult to discriminate between dissociation and unfolding processes (Jaenicke and Lilie, 2000). On the other hand, multimeric enzymes present clear candidate regions for the design of stabilizing mutations, namely, the interfaces between the monomers. It is generally accepted that association of monomers adds to stability meaning that buttressing the interfaces is a feasible strategy for stabilization (e.g. Rahman et al., 1998; Khorkin et al., 1999; Lebbink et al., 1999; Thoma et al., 2000; Arnott et al., 2000; Jaenicke and Lilie, 2000; Kabashima et al., 2001; Clantin et al., 2001; Maeda et al., 2002). The literature contains many reports showing the validity of engineering interfaces between subunits as a strategy to obtain more stable multimeric enzymes. Most of these reports concern cases where residues in a mesophilic enzyme were replaced by corresponding residues from a thermophilic enzyme, but rational interface engineering has also been reported (Sauer et al., 1986; Gokhale et al., 1994; Bjørk et al., 2003a; see below). Flores and Ellington

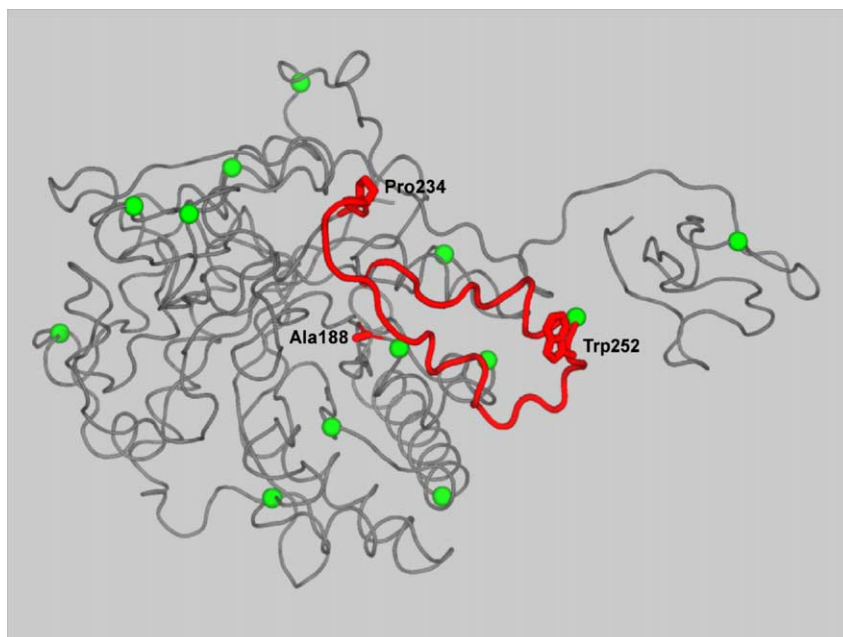


Fig. 4. Mutations in a 499-residue chitinase from *Serratia marcescens*. The picture shows a backbone trace, with the 230–265 surface meander coloured red. Mutated positions (Gly → Ala or Xxx → Pro) are indicated by green balls. The effect of the mutations on half-life at 57 °C were within a factor 0.5 and 1 (mostly close to one, that is, no effect) for all mutations except G188A, A234P and W252P, which increased half-life by factors of 2, 5 and 1.5, respectively. The G188A, A234P double mutant (which is shown here) displayed a 10-fold increase in half-life (Gåseidnes et al., 2003). The side chains of residues 188, 234, and 252 are shown.

(2002) used directed evolution to stabilize a tetrameric beta-glucuronidase, and found that the selected stabilizing mutations accumulated in the interfaces. One of the most stabilizing single point mutations ever reported concerns stabilization of the subunit interface in dimeric *Leishmania* triose phosphate isomerase by a Glu → Gln mutation (Williams et al., 1999).

In some families of multimeric enzymes, the more stable members tend to show a higher oligomerization state than the less stable members (Clantin et al., 2001; Vieille and Zeikus, 2001; Walden et al., 2001; Maeda et al., 2002). The extra interfaces are often characterized by large networks of ionizable side chains, in line with the more general observation that nature often seems to employ networks of salt bridges to obtain high stability (Russell et al., 1994; Yip et al., 1995; Vetriani et al., 1998; Xiao and Honig, 1999; Vieille and Zeikus, 2001). We have recently studied the structures and properties of malate dehydrogenases (MDHs) from green gliding bacteria. These MDHs are tetrameric (Dalhus et al., 2002), in contrast to dimeric MDHs from mesophilic

sources, while not being particularly stable. The extra dimer–dimer interface in these tetrameric MDHs contains a large network of charged residues as well as a contact region with non-charged interactions (Fig. 5). This interface was considered an interesting target for mutations for reasons described above and because the relatively low stability of these MDHs suggested that there might be ample room for improvement. We have conducted mutagenesis studies of one of these enzymes, namely the MDH from *Chloroflexus aurantiacus* (CaMDH). At elevated temperatures, this enzyme becomes irreversibly inactivated via an unfolding process, which, judged by CD measurements, is quite cooperative and quite global in character. The stability of CaMDH can be quantified by deriving apparent  $T_m$  values from CD-unfolding curves.

In initial studies of CaMDH, Glu25 and Asp56 (Fig. 5) were (individually) replaced by their corresponding amides or lysine. All mutations led to decreased stability ( $\Delta T_{m,app}$  upto  $-26.8$  °C at pH 4.4) and the most destabilizing mutation (D56K) also led to

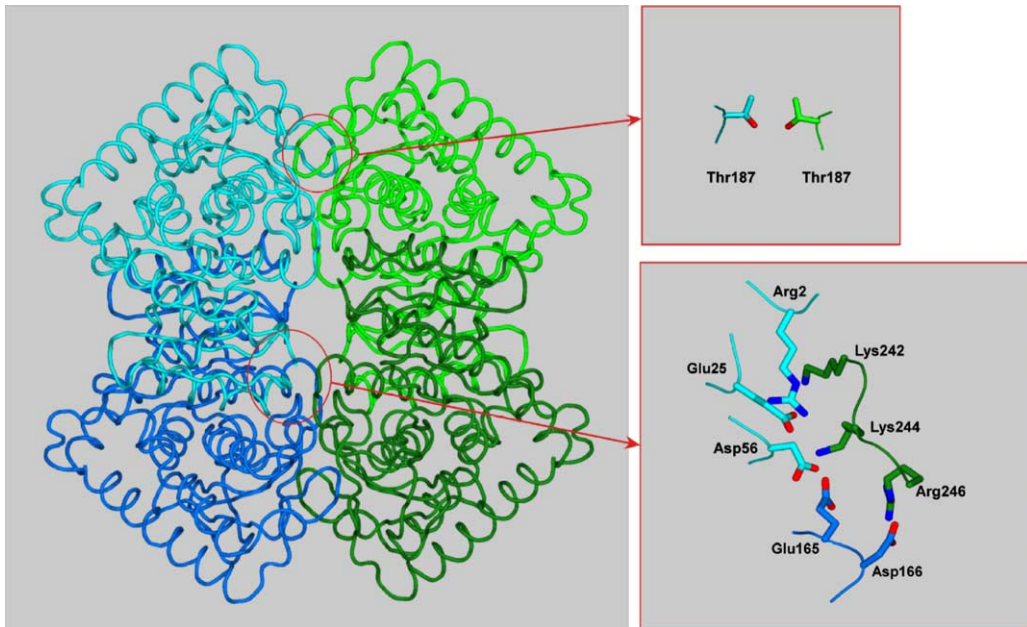


Fig. 5. Tetrameric MDH from *Chloroflexus aurantiacus* and interactions at the dimer–dimer interface. The homo-tetramer consists of two dimers, one in blue and one in green. Some prominent interactions at the dimer–dimer interface are shown in the right panels. Colouring is similar to that in the left panel, except for side chain oxygen and nitrogen atoms, which are coloured red and dark blue, respectively.

loss of tetrameric structure, confirming the importance of the electrostatic network at the dimer–dimer interface (Bjørk et al., 2003b). Interestingly, the stability of the wild-type enzyme as well as the magnitude of the mutational effects were strongly dependent on pH, confirming the idea that stability depends on interactions between titratable groups. The fact that the wild-type enzyme was much more stable at pH 4.4 ( $T_{m,app}$  80.3 °C) than at pH 7.5 ( $T_{m,app}$  67.9 °C) (Bjørk et al., 2003b) and the fact that a  $Cd^{2+}$  ion from the crystallization liquor was found bound close to Glu165 in the crystal structure of wild-type CaMDH (Dalhus et al., 2002) suggested that, at neutral pH, there might be an excess of negative charge at the interface. Indeed, Bjørk et al. (2004) have recently shown that the stability of CaMDH at pH 7.5 can be increased by as much as 24 °C by mutating Glu165, which is located in the vicinity of Glu25 and Asp56 (Fig. 5). It is interesting to note that mutation of residues Glu25 and Asp56 was destabilizing, whereas mutation of the nearby acidic residue Glu165 was clearly stabilizing. Apparently, the electrostatic balance in the network of interactions displayed in Fig. 5 is quite delicate (see Lebbink et al., 1999 for another example).

In an attempt to reinforce the dimer–dimer interface, the SUGCYS option in WHAT IF (Vriend, 1990) was used to automatically detect possible disulfide bridges that could be engineered to link the two dimers in tetrameric CaMDH. Using the standard parameters, only one possible inter-dimer bridge was predicted, namely a bridge between residues 187 (Fig. 5). Upon introduction of the T187C mutation indeed two disulfide bridges were formed per tetramer, yielding an increase in apparent  $T_m$  of 15 °C at pH 7.5 (Bjørk et al., 2003a). Thus, the stability of CaMDH could not only be manipulated drastically by mutating the ionic network, but also by reinforcing the non-ionic network that involves Thr187 (Fig. 5). There are several examples in the literature showing that cross-linking subunits by disulfide bridges in dimeric enzymes is a useful stabilization strategy (Sauer et al., 1986; Gokhale et al., 1994; Kabashima et al., 2001). The work on CaMDH provides an example for a tetrameric enzyme.

In the case of tetrameric CaMDH it was relatively easy to find the “weak link” governing thermal inactivation, namely the subunit interface (the same situation probably applies to most multimeric enzymes). The work on CaMDH and work on other enzymes which is



Fig. 6. Overview of important mutations in *B. licheniformis*  $\alpha$ -amylase. The picture shows a C-alpha trace, where the so-called B domain (residues 101-205) is coloured dark green. Green balls indicate the seven residues (positions 133, 156, 181, 190, 209, 264, 265) that were mutated in a recently described hyperstable seven-fold mutant (Declerck et al., 2003). Dark blue balls indicate two positions (204, 237) whose mutation yielded dramatic destabilization. Light blue and orange balls indicate calcium and sodium ions, respectively. Adapted from Declerck et al., 2003.

referred to above show that major increases in stability can be obtained by engineering this weak link.

### 3.4. $\alpha$ -Amylase

The industrially important stable  $\alpha$ -amylase from *Bacillus licheniformis* has been the subject of protein engineering studies for many years (Joyet et al., 1992; Declerck et al., 2000, 2003 and references therein). Temperature-induced unfolding of this  $\alpha$ -amylase is accompanied by aggregation and precipitation, meaning that only kinetic stability can be measured, e.g. in

terms of half-life or  $T_{50}$  value, as for the TLPs described above. Interestingly, after hundreds of  $\alpha$ -amylase mutants have been made and characterized, the picture emerging is very similar to what has been described for TLPs:

- All mutations that affect thermal stability are located in one particular part of the molecule (the “B domain”; Fig. 6).
- Single mutations in this region can have large destabilizing (upto  $-22^{\circ}\text{C}$  in  $T_{50}$ ) and considerable stabilizing ( $+5^{\circ}\text{C}$  range) effects.

- Combining mutations in this region yielded a hyperstable enzyme showing a 23 °C increase in  $T_{50}$  to a value of 106 °C (Declerck et al., 2003; Machius et al., 2003).
- All stabilizing mutations affect the surface of the protein.

Clearly, the situation for this  $\alpha$ -amylase is very similar as that for TLP-ste, despite the fact that the process that makes unfolding irreversible is aggregation in the former case and autolysis in the latter. In a recent paper Machius et al. (2003) concluded that thermal inactivation of the  $\alpha$ -amylase is driven by local unfolding processes, as has previously been concluded for the thermolysin-like proteases (Vriend and Eijsink, 1993; Eijsink et al., 1995; Kidokoro et al., 1995; Vriend et al., 1998). Machius et al. (2003) use the term “unfolding nucleus” to denote “weak spots” in the protein whose unfolding triggers thermal inactivation.

#### 4. Concluding remarks

Recent literature clearly shows that it is feasible to improve the kinetic stability of proteins by rational design, but that it may be difficult to pinpoint which region of the protein should be focussed on, and which mutation strategy should be used. After having targeted a region for mutagenesis (e.g. by a random mutagenesis approach), the next question is what type of mutations to introduce. Recent literature (e.g. the references quoted above) clearly shows that there are many possibilities to choose from. Entropic stabilization certainly is a good strategy. Another strategy is engineering of surface electrostatics. In this case, one should not focus on individual salt bridges, but rather apply a semi-rational approach where several charges are added or removed in a search for optimal networks (Martin et al., 2001, 2002). Although the contribution of surface electrostatics to protein stability still is not fully understood (e.g. Pace et al., 2000; Strop and Mayo, 2000; Makhatadze et al., 2003), it is clear that these interactions are more important than what was generally believed some 15 years ago.

The wealth of recent reports on stability engineering underlines one clear principle: one cannot generalize the effect of a certain type of mutation on stability. The effect of every single point mutation should always

be viewed in the light of its structural context. Many of the papers quoted above provide examples of this principle (see e.g. Serrano and Fersht, 1989; Blaber et al., 1993; Herning et al., 1992; Hardy et al., 1993; Bogin et al., 1998; Strop and Mayo, 2000; Tollinger et al., 2003; Makhatadze et al., 2003).

With respect to kinetic protein stability, an intriguing question is to what extent the proteins unfold before the irreversible step takes place. On the one hand, the transition state for global unfolding is supposed to resemble the native state (Fersht, 1999), indicating that partial unfolding processes yield only limited loss of structure. On the other hand, there are indications that unfolding in TLPs may involve as much as the complete N-terminal domain (Vriend and Eijsink, 1993; Eijsink et al., 1995 and references therein). Unfortunately, there are few experimental data that help resolve the question of how much unfolding is needed (but see Hori et al., 2000, for an interesting study on 3-isopropylmalate dehydrogenase). Machius et al. (2003) recently pointed out that one should probably not look upon “partial unfolding” as massive loss of structure, but rather as a partial destabilization/flexibilization, which may depend on cooperativity in larger parts of a protein. It is interesting to note that the question of how much unfolding precedes aggregation currently receives much attention in the context of work on amyloid-related diseases (Chiti et al., 2002; Thirumilai et al., 2003).

It is well-known that enzymes isolated from thermophilic organisms tend to be as active at their (high) temperature optima as their counterparts from mesophilic organisms at their (lower) temperature optima (Jaenicke and Bohm, 1998). This has led to the suggestion that there is a trade-off between high stability and high activity, possibly because rigidification of enzymes is needed to attain high stability, whereas conformational flexibility is beneficial for catalysis (Jaenicke and Bohm, 1998). While evolutionary pressure in nature may indeed have promoted such a trade-off (e.g. D’Amico et al., 2003a), recent protein engineering work clearly shows that this trade-off does not exist under non-natural selective pressures (e.g. Van den Burg et al., 1998; Declerck et al., 2003; Bjørk et al., 2003a; Williams et al., 1999). Most importantly, Miyazaki et al. (2000), have shown that the half-life of a psychrophilic protease could be increased 500-fold, without compromising catalysis at low temperatures.

Clearly, the putative inverse correlation between stability and activity is much more complex than previously suggested. One explanation may be that activity requires local flexibility, whereas stability, at least kinetic stability, may be determined by flexibility in regions of the protein that do not affect local flexibility of the active site. In addition to all this, there are conflicting reports in the literature with respect to how rigid hyperstable enzymes really are (Zavodszky et al., 1998; Jaenicke, 2000; Hernandez et al., 2000; D'Amico et al., 2003a,b; Wintrode et al., 2003).

The relationship between thermal stability and the temperature optimum for activity is another intriguing issue (Danson et al., 1996; Daniel et al., 2001). Many enzymes stabilized by protein engineering do have higher temperature optima, but there are now several examples in the literature of proteins showing increased stability, without showing an increase in temperature optimum (e.g. Lebbink et al., 1995; Thomas and Scopes, 1998; Arnott et al., 2000; Bjørk et al., 2003a). Thus, some enzymes seem to have genuine temperature optima (Daniel et al., 2001) that are not always dictated by conformational stability. While conformational stability does not provide a guarantee for thermoactivity it certainly is a prerequisite. Thus, the route to enzymes that are highly active at high temperatures always needs to start with stability engineering.

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