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ADVANCES IN ENGINEERING OF PROTEINS FOR THERMAL STABILITY

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ABSTRACT:

Proteins with increased thermal stability are desirable for many industrial, bioanalytical and pharmaceutical applications. The thermodynamic thermal stability of a protein depends upon the differences in the enthalpy and entropy of the native and denatured states. In addition to thermodynamic stability, the rate of denaturation is also of importance for many applications. A number of model systems have been used for evaluating the importance of the properties that affect thermal stability and a number of novel methods, such as confinement in nanocapsules, have recently been introduced for engineering of proteins for increased thermal stability. The principles and recent applications of protein engineering for thermal stability have been reviewed.

Keywords: Protein engineering, thermal stability

[I] INTRODUCTION

The ability to produce proteins of desired sequence *in vitro* and *in vivo* has led to attempts for production of proteins with increased thermal stability [1]. Proteins of increased thermal stability may be obtained either by protein engineering [2],[3] or by searching for homologs in hyperthermophiles [4]. Many proteins of interest are characteristic of eukaryotes and have no homologs in the thermophilic organisms; protein engineering would be necessary in such cases. Protein engineering may involve directed evolution [5], design from first principles [6] or some combination of these two strategies [7]. The factors affecting the thermal stability of proteins have been identified by comparison of the mesophilic and thermophilic proteins belonging to the same protein family [8]. These insights are useful for optimization of stability of proteins of interest and for design of novel thermostable proteins. A variety of methods, ranging from physics based computational methods [9] to knowledge based approaches are available for prediction of the thermal stability of point mutations [10].

The thermodynamic thermal stability of a protein can be specified by the value of its Tm, the mid-point of the thermal transition. Tm is the temperature at which half of all protein molecules are in native state and the remaining half are in the denatured state [11]. The denatured state is an ensemble of many conformations and has high conformational entropy [12]. Higher values of Tm indicate an increase in thermal stability. Molecules in the denatured state are more likely to be affected by proteolysis, adsorption, aggregation and precipitation. These processes are often irreversible and may lead to a continuous decrease in the concentration of the protein in the native state. For many industrial applications, the kinetic instability, which is a measure of the rate of conversion of native state into other forms (often irreversible), is also of relevance [13]. The kinetic instability is either specified by the half-life time or a rate constant (k_{unf}). $dN/dt = -k_{unf}N^a$

'a' is the order of the reaction, it is often one; however, for many irreversible processes, such as aggregation and precipitation, 'a' may be much larger than one. Therefore, conversion of even a small fraction of the native state may trigger

irreversible processes and accelerate the denaturation process.

The thermodynamic stability may be increased by increasing the free energy difference between the native and denatured states – this may be achieved by stabilization of the native state or by destabilization of the denatured state [12]. The kinetic stability of a protein may be increased by increasing the free energy difference between the native state and the transition state [14].

The free energy of the native state may be lowered by stabilization of the native state. This can be achieved by optimization of electrostatic and van der Waals interactions between the atoms, by minimization of strain, by optimization of hydrogen bonding networks and by optimization of the spatial distribution of polar and hydrophobic residues [12],[15].

The free energy of the denatured state can be increased by decreasing the conformational entropy [16]. A variety of strategies are available for reducing the entropy of the denatured state: cross-linking, post-translational modifications, immobilization, adsorption and confinement in nanoparticles. The effects of changes in conformational entropy can often be predicted qualitatively or by using calculations based on statistical mechanics [17],[18]; however, the total change in entropy (which includes includes hydration and other effects) is difficult to predict and requires considerable computational resources even for small changes in protein sequence [9].

The free energies of the native and denatured states can be rationally engineered based on knowledge of the native structure of the protein and the knowledge regarding the statistical distributions of conformations in the denatured states [19]. Rational manipulation of the free energy of the transition state is more difficult because of the limited structural knowledge regarding the nature of the transition state involved in protein unfolding [20]. The kinetic rate constant method, which compares the rates of unfolding in the wild-type protein with that in the mutant with an extra cross link provides qualitative information regarding the structure in the transition state ensemble [21],[16]. Methods based on phi-value analysis provide information for characterization of the transition state in protein unfolding [22]. In phi-value analysis, a series of mutants are produced; for each mutant the free energy difference between the transition state and the native state (ΔG_{ts}) and the free energy difference between the denatured state and the native state (ΔG_{unf}) are measured. The ratio of these two ΔGs is the phi-value for the mutant. The magnitude of the phi-value is a measure of the extent to which the transition state retains native structure, in the vicinity of the mutated residue [14]. These data have been used for engineering of proteins for increased thermal stability [23]. A complimentary method for characterization of the transition state is the psi-value method [24]. The psi-value method compares the rate of unfolding as a function of metal ion concentration in mutants stabilized by formation of a cross-link. The non-covalent cross-link is formed by chelation of two histidine residues to a metal ion. In addition, combined approaches that use molecular modeling and experimental data from mass spectroscopy and site-directed mutagenesis provide insights regarding the factors affecting the kinetic thermal stability of proteins [25].

[II] METHODS

2.1. Site directed mutagenesis

Site directed mutagenesis was first described by Gillam et al [26]. The Kunkel method of site directed mutagenesis is often used to introduce a mutation at the desired site without phenotypic selection [27]. An oligonucleotide containing the desired changes can be incorporated into a covalently closed complimentary strand circle using a uracil containing template; this confers a strong selective advantage over the template containing the unaltered genotype [27]. A variety of PCR based site-directed mutagenesis methods

have been introduced and these have the advantage that the template may be either single or double stranded, linear or circular in form [28].

2.2. Segment/Domain deletion and ligation

Thermostability of proteins may be improved by ligation to a thermostable domain [29], which may be a replacement for one of the domains in the protein or a completely new domain. Similarly, deletion of certain segments may increase the thermostability of the protein by reduction of the entropy of the denatured state [30].

2.3. Directed evolution

A number of molecular biological methods are available for directed evolution of proteins [5], e.g. random mutagenesis [31], DNA shuffling and chimeragenesis [32],[33], nonhomologous recombination [34], etc. Rational design may also be combined with directed evolution. High-throughput screening for thermal stability based on differential light scattering and differential scanning fluorescence requires smaller amounts of material; in addition, lower concentrations can be used, compared to conventional differential scanning calorimetric methods [35],[36]. The emergence of high-throughput screening technologies for thermal profiling will enable screening of larger libraries [37].

Error prone PCR can be used to produce a large number of variants [38], which are then selected for by using a suitable screen. The surface display technique has been of particular use for rapid screening and amplification [39],[40],[41]. Display technologies using bacteriophage as well as yeast are now available.

DNA shuffling, at the segment or domain level, provides a rapid method for generation of a large number of variants, that have a high likelihood of retaining the native activity and the diversity is helpful for selection of variants that have high thermostability while retaining the native activity. Mutants produced by site-specific mutation and error prone PCR may be combined with DNA shuffling [42], The yeast homologous recombination system has been found to be very efficient for shuffling of mutations among a large number of parents [33].

SCHEMA is an algorithm for optimization of the libraries produced by recombination to generate chimeras [43]. The computational algorithm identifies structural elements that can be swapped without disrupting the integrity of the protein structure.

2.4. Novel thermostable scaffolds

Thermostable proteins with the desired specificity and activity may be obtained by grafting of a few segments that are essential for binding or activity on to a scaffold that has been chosen or designed specifically for high thermostability [44],[45], [46], [47].

2.5. Proteins with novel non-native amino acids

In vitro transcription and translation systems are able to incorporate non-native amino acids into the protein chain, effectively extending the genetic code [48]. The codon specifying the amino acid to be changed is altered to one of the three stop codons, and is used to specify the novel amino acid. The in vitro translation system has an additional amino-acyl t-RNA with an anti-codon complimentary to the stop codon that has replaced the codon coding for the native amino acid; and the 5' end of the tRNA is acylated to the non-native amino-acid of interest. Amino-acids that lower the entropy of the denatured state due to lower conformational flexibility are of particular interest for engineering proteins with enhanced thermal stability [49]. Recent advances in the use of orthogonal amino-acyl tRNA synthetase/tRNA pairs have enabled the addition of non-native amino-acids to E. coli, yeast and mammalian cells [50].

Peptide nucleic acids (PNA) have high thermal and chemical stability and are attractive candidates for molecular diagnostics. A semi-automated method for synthesis of PNA and PNA-peptide conjugates has been reported recently – this may enable high-throughput screening of PNA-peptide conjugates as diagnostic probes or therapeutic drugs [51].

2.6. Chemical modification and additives for stabilization of proteins

Chemical methods for selective modification of cysteine, histidine, lysine, arginine, tyrosine, aspartic and glutamic acids, asparagine and glutamine are available and have been reviewed recently [52]. A number of bifunctional reagents for introducing intra- and intermolecular cross-

links are available; these are often able to increase thermal stability with little or no change in the specificity and efficiency of the enzymatic properties because they involve residues on the surface. The reagent design and and cross-linking protocols have been recently reviewed [53].

It has been demonstrated that PEGylation increases the thermal stability of small pharmaceutical proteins [54]. The stability of proteins may also be improved by covalent protein immobilization [55]. Brady and Jordan have reviewed recent advances in enzyme immobilization [56].

Binding of ligands alters the thermal stability and this effect can be used for stabilization of proteins [57],[58].

Microcalorimetry can be used as a rapid screening tool for assessment of the solution stability of high molecular weight pharmaceutical protein preparations [59]. Thermofluor based high-throughput optimization methods are now being used for rapid evaluation of stabilizing and destabilizing effects of additives [60].

2.7. Confinement in nanoparticles

Entrapment of proteins in nanoparticles increases the thermal stability of the protein by decreasing the entropy of the unfolding state and thereby increasing the free energy of the denatured state. The nanoparticles of interest might either be produced by genetic engineering of chaperones such as Hsp16.5 [61] or by biomimetic silicification [62]. Nanospheres may be generated by addition of silicic acid to positively charged dendrimers of polyamidoamine (PAMAM). The silicic acid deposits on the surface, and hardened shells are formed upon curing [62].

[III] RESULTS

The results of recent attempts of protein engineering for increasing the thermal stability of proteins that have the potential for applications in industrial and pharmaceutical applications are summarized below (Table 1). These results indicate that it is now possible to use protein engineering to obtain significant increases in thermal stability of proteins, while retaining the native function.

3.1. Enzymes of industrial significance

The ability to work at high temperatures is advantageous in many industrial applications [13]. Increased temperature leads to lower viscosity and ease of mixing; in addition, the loss of activity of enzymes that may be present in feedstock or as impurities in the enzyme preparation ensures that the enzyme of interest can function without interference from the action of other enzymes. Furthermore, increase of temperature can destabilize some of the substrates such as ligno-cellulosic materials and enable more effective processing. Therefore, enzymes of industrial significance have been the target of a number of attempts for engineering for increased thermal stability [13], e.g., invertase [73], amylase [83],[84], glucanase [65], xylanase [85], cellulase [86], tyrosine-phenol lysase [68].

3.2. Protein engineering for bioanalytical applications

Thermal stability is of critical importance for the DNA polymerases used in Polymerase chain reaction. A number of DNA polymerases from hyperthermophilic microbes are currently in use; the thermal stability, specificity (low error rate) and processivity of these enzymes have been the target of many protein engineering studies, which aim to optimize all three of these essential properties simultaneously.

The sensing elements of Biosensors are often enzymes or receptors. The kinetic stability of these proteins is critical to maintain reproducible response and to avoid frequent recalibration of these devices. Immobilization and other methods of protein engineering are being used for increasing the stability of proteins used as sensor elements in Biosensors. For example, the kinetic stability of glucose oxidase, an enzyme used in the glucose biosensor, has been increased by immobilization [87].

The cystine-knot based scaffold can be used for the development of peptide based in-vivo molecular diagnostics. The cystine-knot scaffold can be functionalized by bioactive loops designed to bind target proteins; these designed miniproteins are being evaluated in animal models for tumor imaging [88].

3.3 Protein engineering for pharmaceutical applications

The factors affecting the stability of porteins used in pharmaceutical applications have been studied thoroughly and a number of methods have been introduced for stabilization of protein pharmaceutical preparations [89]. The kinetic stability is in general of greater importance in pharmaceutical applications than the thermodynamic thermal stability. Kinetic stability is required for storage of pharmaceutical preparations, preferably at room temperature. In addition, the kinetic stability is related to the residence time of the protein in the human body. For example, granulocyte colony stimulating factor (GCSF) was ligated to a collagen like protein – this led to an increase in thermal stability as well as a decrease in the plasma clearance rate and a concomitant ability to stimulate increase in while blood cells for a longer duration than native GCSF [79].

[IV] DISCUSSION

The high specificity of the reaction catalyzed by enzymes is a critical factor for the choice of enzymes in many industrial processes. Any alteration of the protein sequence has the potential to cause undesirable changes in protein function such as altered specificity. Changes in specificity of an enzyme may necessitate a complete alteration of the bioprocess and downstream purification and quality control stages in a production line. This may be less important for proteins engineered by post-translational modifications; however, even post-translational modifications on the surface have the potential to alter specificity and may significantly alter the solubility, adsorption properties and immunogenicity. In view of these limitations of traditional methods of protein engineering, the increase in thermostability achievable by confinement of proteins in hollow nanoparticles is of particular interest. The specificity of the proteins for small molecule substrates and ligands would be expected to remain unaltered due to confinement. Therefore, this method has the potential for increasing thermal stability without requiring a trade-off in terms of specificity or other functional properties of interest. In pharmaceutical applications, the nanoparticle surface would shield the protein of interest from the host's immune system, if the carrier is designed to be non-immunogenic. In addition, the same carrier may be used for a variety of proteins, which may lead to considerable reduction in the costs of clinical and pre-clinical testing of proteins used for pharmaceutical purposes. However, stabilization by confinement in nanoparticles is not appropriate for pharmaceutical proteins whose

function involves binding to macromolecules or large cell surface antigens; because the enclosing surface of the nanoparticle surface would shield the protein of interest from its macromolecular target.

[V] CONCLUSION

The methods that are currently being used for engineering of proteins for enhanced stability have been briefly reviewed. The principles that guide the design and engineering of proteins, and recent applications of protein engineering for enhancement of thermal stability of proteins that have the potential for industrial applications, have also been reviewed. Engineering of proteins for improved thermal stability is emerging as an effective alternative to search for proteins with similar activity in hyperthermophiles; in addition, it is the only option available if the protein of interest has no homologs in the thermophilic organisms.

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[Table-1]. Recent applications of protein engineering or thermal stability

Note: Applications of protein engineering for thermal stability in the period preceding 2008 have been reviewed by O'Fagain[1].

Protein	Method	Result
Protease from Aspergillus oryzea [63]	Encapsulation within biomimetically generated silicate nanospheres	Enhanced thermostability
Pyranose 2-oxidase from Trametes multicolor [64]	Designed triple mutant	Increase half life from 7.7 min to 10 hours (at 60C)
Endo beta-glucanase EgI499 from Bacillus subtilis JA18 [65]	Deletion of C-terminal region	Increaes in half life from 10 to 29 mins at 65C
Lipases from Yarrowia lipolytica [41]	Surface display on Saccharomyces cerevisiae	Increased thermal stability and activity
Alkaline protease from Aureobasidium pullulans HN2-3 [66]	Surface display on yeast Yarrowia lipolytica	Increased thermal stability and decresed pH stability
Xylanase XT6 from Geobacillus stearothermophilus [67]	Directed evolution and site-directed mutagenesis	52x increase in thermal stability, kopt increase by 10C, catalytic efficiency increase by 90%
Tyrosine phenol-lyase from Symbiobacterium toebi [68]	Directed evolution (random mutagenesis, reassembly and activity screening)	Improved thermal stability and activity (Increase in Tm upto 11.2 C)
Phytase from Penicilium [69]	Random mutation and selection	Increased thermal stability
Arabinose isomerase [70]	Immobilization on aminopropyl glass modified with glutaraldehyde	Kinetic thermal stability increase by 138 fold.
Papain [71]	Immobilization on cotton fabric with anhydrides	Improved thermal stability and stability to alkali and detergent
Cellobiase [72]	Immobilization with chitosan-alginate	Increased thermal stability
Invertase [73]	Inter- and intra-molecular cross-linking with diisocynate reagents	First order thermal denaturation rate constant reduced from 1.567 min-1 for native to 0.5 min-1
Adenylated kinase [74]	Improved configurational entropy by structural entropy optimization	Improved thermal stability
L-Asparaginase [75]	In vitro directed evolution	Increase in half-life from 2.7 to 159.7 hours
Human glucocorticoid receptor ligand binding domain (hGR-LBD) [76]	Random mutagenesis and high-throughput screening using fluorescent activated cell sorting with enhance green fluorescent protein as a reporter	Thermal stability upto 8C greater than wild type.
MDM2 [77]	Site-directed mutagenesis	Increased thermal stability
HIV 4E10 epitope [78]	Flexible backbone remodeling and resurfacing	Improved thermal stability
Human epidermal growth factor specific affibodies [45]	Graft antigen-binding segments from antibody to human EGF (HER-2) onto a designed thermostable three-helix bundle	HER-2 binding ability retained after heating to 90C, three times. Binding constant 76pm.
Granulocyte colony stimulating factor [79]	Fusion with gelatin like protein polymer	Increases thermal stability, slower plasma clearance rate,
Heme peroxidase from cinereus [80]	In silico design using rosettadesign and site-specific mutation	2/8 of designed enzyme were more stable than wild type.
Cytochrome P450 [81]	Rational mutagenesis	Increased thermal stability
Human erythropoeitin [82]	Polyethyleneglycosylation	Increased thermal stability, prolonged circulating half-life in rats